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(54) Title: HETEROARYLCARBOXAMIDE COMPOUNDS ACTIVE AGAINST PROTEIN TYROSINE KINASE RELATED DISORDERS

(57) Abstract

The present invention relates to novel heteroarylcarboxamides which modulate the activity of protein tyrosine kinases and are expected to be useful in the treatment of abnormal protein tyrosine kinase activity driven disorders, to methods for the treatment of inappropriate FGFR activity related disorders with the heteroarylcarboxamide, N-(4-trifluoromethyl-phenyl)-5-methylisoxazole-4-carboxamide, and to the treatment of solid tumor cancers, especially glioblastoma and astrocytoma, with a combination of a nitrosourea, preferably BCNU (carmustin) and N-(4-trifluoromethyl-phenyl)-5-methylisoxazole-4-carboxamide.

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DESCRIPTION

Heteroarylcarboxamide Compounds Active Against Protein Tyrosine Kinase Related Disorders

Introduction

The present invention relates generally to organic chemistry, biochemistry, pharmacology, medicine and cancer therapy. More particularly, it relates to heteroarylcarboxamide compounds and their physiologically acceptable salts which modulate the activity of protein tyrosine kinases and, as a result, are expected to exhibit a salutary effect against disorders related to abnormal protein tyrosine kinase activity including cancer. Novel chemotherapeutices comprising a heteroarylcarboxamide in combination with known chemotherapeutic agents such as the nitrosoureas are also described herein.

Background of the Invention

The following is offered as background information only and is not admitted to be prior art to the present invention.

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. Growth factor receptors ("Gfrs") are an important part of the signal transduction pathway. Gfrs are cell-surface proteins. When bound by a growth factor ligand, Gfrs are converted to an active form which interacts with proteins on the inner surface of a cell membrane. As the result of this interaction, one of the key biochemical mechanisms of signal transduction is initiated; i.e., the reversible phosphorylation of various proteins within the cell. This phosphorylation of intracellular proteins causes the formation inside the cell of complexes with a variety of cytoplasmic signaling molecules that, in turn, effect numerous cellular responses such as cell

division (proliferation), cell differentiation, cell growth, expression of metabolic effects on the extracellular microenvironment, etc. For a more complete discussion, see Schlessinger and Ullrich, Neuron, 9:303-391 (1992). See also, Posada and Cooper, Mol. Biol. Cell., 3:583-392 (1992) and Hardie, Symp. Soc. Exp. Biol., 44:241-255 (1990).

The molecules which effect the phosphorylation of proteins are called protein kinases ("PKs"). One of the classes of PKs which is of particular importance to the present invention phosphorylates proteins on the alcohol (-OH) moiety of serine, threonine and tyrosine residues in eukariotic cells. These PKs fall essentially into two groups, those specific for phosphorylating serines and threonines, and those specific for phosphorylating tyrosines. The protein tyrosine kinases ("PTKs") can be further divided into receptor PTKs or "RTKs" and non-receptor PTKs, sometimes referred to as "cellular tyrosine kinases" or "CTKs."

The RTKs are comprised of an extracellular glycosylated ligand binding domain, a transmembrane domain and an intracellular cytoplasmic catalytic domain that can phosphorylate tyrosine residues on proteins. CTKs are entirely intra-cellular and do not contain extracellular and transmembrane domains.

PTKs play an important role in the control of cellular processes including proliferation, differentiation, migration and survival. It is clear from numerous studies (q.v., infra) that the activity of PTKs must be tightly controlled in normal cells and healthy tissue, as mutations resulting in overactivity and/or overexpression of PTKs cause diseases that are associated with excessive cell growth and proliferation such as, significantly, cancer. At the other end of the spectrum, mutations which result in reduction or loss of activity can cause embryonal lethality or developmental disorders.

The RTKs comprise one of the larger families of PTKs and have diverse biological activity. At present, at least nineteen (19) distinct subfamilies of RTKs have been identified. One such subfamily is the "HER" family of RTKs, which include EGFR (epithelial growth factor receptor), HER2, HER3 and HER4. These RTKs consist of an extracellular glycosylated ligand binding domain, a transmembrane domain and an intracellular cytoplasmic catalytic domain that can phosphorylate tyrosine residues on proteins. One well-known example of the apparent involvement of PTKs/RTKs in cellular disorders is the association of Her2 over-expression with breast cancer (Slamon, et al, Science, 244:707 (1989).

Another RTK subfamily consists of insulin receptor (IR), insulin-like growth factor I receptor (IGF-1R) and the insulin receptor related receptor (IRR). IR and IGF-1R interact with insulin, IGF-I and IGF-II to form a heterotetramer of two entirely extracellular glycosylated α subunits and two β subunits which cross the cell membrane and which contain the tyrosine kinase domain.

A third RTK subfamily is referred to as the platelet derived growth factor receptor ("PDGFR") group, which includes PDGFRα, PDGFRβ, CSFIR, c-kit and c-fms. These receptors consist of glycosylated extracellular domains composed of variable numbers of immunoglobin-like loops and an intracellular domain wherein the tyrosine kinase domain is interrupted by unrelated amino acid sequences.

Another group which, because of its similarity to the PDGFR subfamily, is sometimes subsumed in the later group, is the fetus liver kinase ("flk") receptor subfamily. This group is believed to be made of up of kinase insert domain-receptor fetal liver kinase-1 (KDR/FLK-1), flk-1R, flk-4 and fms-like tyrosine kinase 1 (flt-1).

Finally, the fibroblast growth factor ("FGF") family of PTKs contains at least four distinct members: fibroblast growth factor receptor 1 (FGFR1, also called Flg and Cek1), FGFR2

(also called Bek, Ksam, KsamI and Cek3), FGFR3 (also called Cek2) and FGFR4. They share a common structure consisting of, in the mature protein, one or more immunoglobulin-like (IgG-like) loops flanked by characteristic cysteines, a hydrophobic transmembrane domain and a intracellular domain containing a catalytic region that is split by a short insert; See Ullrich and Schlessinger, Cell,61:203 (1990). Additional diversity in this receptor family is generated by alternative RNA splicing within the extracellular domain (Jaye, et al., Biochem. Biophys. Acta 1135:185, 1992; Johnson and Williams, Adv. Cancer Res. 60:1, 1993).

Fibroblast growth factors and their receptors, the FGFRs, play an important role in angiogenesis, wound healing, embryonic development, and malignant transformation (Basilic and Moscatelli, Adv. Cancer Res. 59:115, 1992). For instance, FGFRs have been implicated in the induction of angiogensis (Folkman and Klagsbrun, Science 235:442, 1987; Klagsbrun and D'Amore, Annu. Rev. Physiol. 53:217, 1991), suggesting that inappropriate FGFR activity may contribute to human disorders such as diabetic retinopathy, macular degeneration, rheumatoid arthritis, atherosclerosis and tumor neovascularization. In fact, several members of the FGFR family were originally isolated as oncogenes (Delli Bovi, et al., Cell 50:729, 1987) suggesting a close relationship between FGFRs and malignant transformations. FGFRs have been directly associated with a number of cancers including oral and salivary gland cancer (Myoken, et al., Int. J. Cancer 65(5):650, 1996) prostate cancer (Nakahara, et al., Nishinihon J. Urology 58(4):310, 1996; Nakamoto, et al., Cancer Res. 52(3):571, 1992), breast cancer (Penault-Llorca, et al., Int. J. Cancer 61(2):170, 1995; Adnane, et al., Oncogene 6(4):659, 1991; Wellstein and Lippman, Molecular Foundations of Oncology, Broder, S. ed., pgs 403 - 418, 1991), pancreatic cancer (Ohta, et al., Br. J. Cancer 72(4):824, 1995; Yamanaka, et al., Cancer Res. 53:5292, 1993; Friess, et al., Chirug 65(7):604, 1994), ovarian cancer

(Crickard, et al., Gynecol. Oncol. 55(2):277, 1994), brain cancer (Yamaguchi, et al., PNAS 91(2):484, 1994; Takahashi, et al., FEBS Letts. 28891):65, 1991), kidney cancer (Nanus, et al., Proc. Annu. Meet. Am. Assoc. Cancer Res. 32:A428, 1991), bladder cancer (Allen and Maher, J Cell. Physiol. 155(2):368, 1993), Kaposi's sarcoma (Jian, et al., Cancer 72(7):2253, 1993), and colon cancer (New and Yoeman, J. Cell. Physiol. 150(2):320, 1992). Moreover several groups have reported an association between abnormal expression of an FGFR with tumor aggression, invasiveness and metastasis.

Mutations in human FGFR genes have been also implicated in a variety of human skeletal disorders. For example, mutations resulting in the constitutive (ligand-independent) activity of FGFR3 play a role in long bone disorders and cause several clinically related forms of dwarfism including achondroplasia, hypochondroplasia and the neonatal-lethal thanatophoric dysplasia (Shiang, et al., Cell 78:3,35, 1994; Rousseau et al., Nature 371:252, 1994; Bellus et al., Nature Genet. 10:357, 1995; Tavormina, et al., Nature Genet. 9:321, 1995; Webster and Donoghue, EMBO J. 15:520, 1996; Webster, et al., Mol. Cell Biol. 16:4081, 1996; Naski, et al., Nature Genet. 13:233, 1996).

A more complete listing of the known RTK subfamilies is described in Plowman et al., DN&P, 7(6):334-339 (1994) which is incorporated by reference, including any drawings, as if fully set forth herein.

At present, over 24 CTKs in 11 subfamilies (Src, Frk, Btk, Csk, Abl, Zap70, Fes, Fps, Fak, Jak and Ack) have been identified. The Src subfamily appear so far to be the largest group of CTKs and includes Src, Yes, Fyn, Lyn, Lck, Blk, Hck, Fgr and Yrk. For a more detailed discussion of CTKs, see Bolen, Oncogene, 8:2025-2031 (1993), which is incorporated by reference, including any drawings, as if fully set forth herein.

Both RTKs and CTKs have been implicated in a host of pathogenic conditions including, significantly, cancer.

Others pathogenic conditions to which RTKs and CTKs have been linked include, without limitation, psoriasis, hepatic cirrhosis, diabetes, atherosclerosis, arterial restenosis, wound scarring, kidney sclerosis and a variety of other renal disorders.

With regard to cancer, two of the major hypotheses advanced to explain the excessive cellular proliferation that drives tumor development relate to functions known to be PTK regulated. That is, it has been suggested that malignant cell growth results from a breakdown in the mechanisms that control cell division and/or differentiation. It has been shown that the protein products of a number of proto-oncogenes are involved in the signal transduction pathways that regulate cell growth and differentiation. These protein products of proto-oncogenes include the extracellular growth factors, transmembrane growth factor PTK receptors (RTKs) and cytoplasmic PTKs (CTKs), discussed above.

In view of the apparent link between PTK-related cellular activities and a number of virulent human disorders, it is no surprise that a great deal of effort is being directed at finding ways to modulate PTK activity. Some of these have involved biomimetic approaches using large molecules patterned on those involved in the actual cellular processes (e.g., mutant ligands (U.S. App. No. 4,966,849); soluble receptors and antibodies (App. No. WO 94/10202, Kendall and Thomas, Proc. Nat'l Acad. Sci., 90:10705-09 (1994), Kim, et al., Nature, 362:841-844 (1993)); RNA ligands (Jelinek, et al., Biochemistry, 33:10450-56); Takano, et al., Mol. Bio. Cell 4:358A (1993); Kinsella, et al., Exp. Cell Res. 199:56-62 (1992); Wright, et al., J. Cellular Phys., 152:448-57) and tyrosine kinase inhibitors (WO 94/03427; WO 92/21660; WO 91/15495; WO 94/14808; U.S. Pat. No. 5,330,992; Mariani, et al., Proc. Am. Assoc. Cancer Res., 35:2268 (1994)).

More recently, attempts have been made to identify small molecules which act as PTK inhibitors. For example, bis-monocylic, bicyclic and heterocyclic aryl compounds (PCT WO 92/20642), vinylene-azaindole derivatives (PCT WO 94/14808) and 1-cyclopropyl-4-pyridylquinolones (U.S. Pat. No. 5,330,992) have been described as tyrosine kinase inhibitors. Styryl compounds (U.S. Pat. No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Pat. No. 5,302,606), quinazoline derivatives (EP App. No. 0 566 266 A1), selenaindoles and selenides (PCT WO 94/03427), tricyclic polyhydroxylic compounds (PCT WO 92/21660) and benzylphosphonic acid compounds (PCT WO 91/15495) have all been described as compounds which are PTK inhibitors and therefore should be useful in the treatment of cancer.

The broad range of human disorders which appear to implicate PKs has clearly attracted the attention of the pharmaceutical/bio-technical research community; in particular the increasingly apparent nexus of PKs to cancer and the possibility of specific, directed treatments of cancer, perhaps even the prevention of the disease through prophylactic treatment of abnormal PK activity, has generated a great deal of interest. This is not surprising in that cancer continues to be one of the leading causes of death in human beings. The majority of cancers are solid tumor cancers such as ovarian cancer, colon cancer, brain cancer, prostate cancer, lung cancer, Kaposi's sarcoma and skin cancer. Among the most virulent of the solid tumor cancers are the brain cancers, in particular primary intra-axial brain cancers such as glioblastoma multiforme, astrocytoma, anaplastic astrocytoma, ependymoma, oligodendroglioma, medulloblastoma, meningioma, sarcoma, hemangioblastoma and pineal parenchymal cancer.

The primary modes of treatment of brain cancers, as with all solid tumor cancers, are surgery, radiation therapy and chemotherapy, separately and in combination. Despite advances in all three approaches, the survival rate of patients diagnosed with primary malignant brain tumors remains

depressingly stagnant; i.e., significant changes in long-term survival have not been realized. Charette, J. L., Neuro-ncology, 7(1):135 (1995).

A malignancy within the brain presents particular difficulties not associated with other cancers. That is, the "blood-brain barrier" prevents penetration of many types of molecules into the brain parenchyma. Other complications involve the lack of lymphatic drainage within the brain to effect the removal of cellular wastes and toxins and the fixed space (the cranium) within which the tumor is located, which can give rise to increased intracranial pressure due to the fact that some tumors swell when they are subjected to chemotherapy. Charette, ibid.

At present, the drugs generally regarded as having the best activity against brain tumors are alkylating agents which have been shown to display a directly proportional dose-response relationship. The alkylating agents are cytotoxic; that is, they kill cancer cells, usually by affecting DNA synthesis or function.

Within the realm of alkylating agents, the nitrosoureas are the agents of choice for treatment of brain tumors due to their ability to cross the blood-brain barrier. N,N'-bis(2-chloroethyl)-N-nitrosourea or BCNU is the most active of the nitrosoureas against malignant gliomas, especially at high dosages. However, BCNU at high dosage; e.g., in excess of 800 mg/m^2 (cancer drug dosages are generally reported in either mg/kg (milligrams of drug per kilogram body weight) or mg/m^2 (milligrams of drug per square meter of body surface area), the latter being the predominant format for most cancer chemotherapeutic applications), even with marrow rescue, results in unacceptably high morbidity and mortality, often associated with pulmonary and/or neurologic toxicities. Finlay, J. L., Bone Marrow Transplantation, 18 (suppl. 3):S1-S5 (1996). Multi-drug combination therapy at lower dosages, which might ameliorate this situation has been explored by numerous

groups. See, e.g, Kiu, M. C., et al., Neuro-Oncology, 25(3):215-20 (1995); Aas, A. T., et al., British Journal of Neurosurgery, 8(2):187-92 (1994); Plowman, J., et al., Cancer Research, 54(14):3793-9 (1994); Wedge, S. R., et al., British Journal of Cancer, 73(9):1049-52 (1996); Kurpad, S. N., et al., Cancer Chemotherapy and Pharmacology, 39(4):307-16 (1997); and Hochberg, F., et al., Neuro-Oncology, 32(1):45-55 (1997). However, these studies have focused on combinations of cytotoxic drugs and no such combination has yet proven entirely satisfactory. Nevertheless, chemotherapy remains one of the foundations of cancer treatment and the search for new and better drugs and combinations thereof continues unabated.

Summary of the Invention

Our own efforts to identify small organic molecules which modulate PTK activity and which, therefore, should be useful in the treatment and/or prevention of disorders driven by abnormal PTK activity, has led us to the discovery, which comprises one aspect of this invention, that the compound N-(4-trifluoromethylanilino)-5-methylisoxazole-4-carboxamide, a heteroarylcarboxamide described initially by Hirth, et al., WO 95/19169, published July 20, 1995 and Bartlett, et al., WO 91/17748, published November 28, 1991, both of which are incorporated by reference as if fully set forth herein, and known commonly as leflunomide, HWA 486 or A10 (and which shall be referred to herein as MCTA), is an effective modulator of select PK, notably the RTKs PDGFR and FGFR, activity. This, in turn, led us to a family of novel heteroarylcarboxamide compounds which likewise modulate PK activity and which comprise another aspect of this invention.

We have further discovered a surprisingly effective chemotherapeutic combination of MCTA with known chemotherapeutic agents, primarily the nitrosoureas such as BCNU, in the treatment of solid tumor cancers, especially the extremely virulent brain cancer, glioblastoma.

Thus, in one aspect, the present invention relates to a method for the treatment of diseases related to inappropriate FGFR activity by administering to a patient in need of such treatment a therapeutically effective amount of the heteroarylcarboxamide, N-(4-trifluoromethylanilino)-5-methylisoxazole-4-carboxamide ("MCTA") in a pharmacological composition. In particular, the FGFR-related disease is cancer.

In another aspect, the present invention relates to novel heteroarylcarboxamides which modulate the activity of PKs, in particular RTKs, especially PDGFR and FGFR. In addition, the present invention relates to the preparation and use of pharmacological compositions of the disclosed compounds and their physiologically acceptable salts in the treatment and prevention of PTK driven disorders such as, significantly, cancer.

Finally, the present invention relates to the treatment of solid tumor cancers, especially glioblastomas, using combinations of MCTA with known chemotherapeutic agents such as the nitrosoureas, exemplified herein by BCNU.

As used herein, a "heteroarylcarboxamide" refers to a compound having the general structure shown in Formula 3.

A "pharmacological composition" refers to a mixture of one or more of the compounds described herein, or a physiologically acceptable salt thereof, with other chemical components, such as pharmaceutically acceptable carriers and excipients. The purpose of a pharmacological composition is to facilitate administration of a compound to an organism.

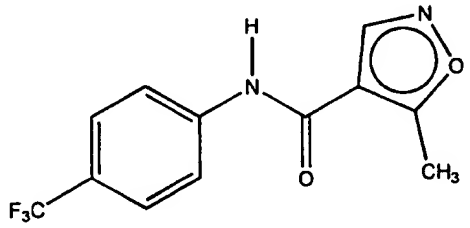
As used herein, "physiologically acceptable carrier" or "pharmaceutically acceptable carrier" are used interchangeably to refer to a carrier or diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

An "excipient" refers to an inert substance added to a pharmacological composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Detailed Description of the Invention

The Compounds

4-(N-(4'-trifluoromethylphenyl)carboxamido)-5-methyl-isoxazole ("MCTA,"Formula 1), to which the compounds of the present invention are structurally related, is a compound currently in clinical testing, based on its ability to inhibit unwanted cell proliferation, both as an immunosuppressive and a cancer drug. MCTA is believed to be metabolized in serum such that the isoxazole ring is converted to an open form called in the literature A771726 and having the chemical structure shown in Formula 2.



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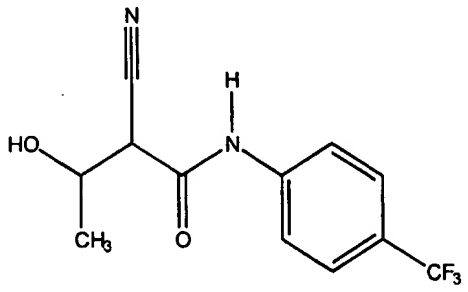


2

Several early reports suggested that MCTA was capable of inhibiting tyrosine kinase signaling (Bartlett, et al., Agents and Actions,32:10 (1991), Mattar, et al., FEBS Lett., 334:161 (1993), Xu, et al., J. Biol. Chem., 270:12398 (1995)). More recently Cherwinski, et al. reported that MCTA has no effect on tyrosine kinase signaling but inhibits proliferation by inhibiting DNA replication and entry of cells into the M-phase of the cell cycle (J. Pharma. and Exp. Thera., 272:460 (1995)). Subsequent reports have suggested that the activity of MCTA is solely due to the ability of A771726 to inhibit pyrimidine biosynthesis by inhibiting a key enzyme in that process, dihydroorotate dehydrogenase (DHOD) (Nair, et al., Imm. Lett., 47:171 (1995), Greene, et al., Biochem. Pharma., 50:861 (1995), Cherwinski, et al., Inflamm. Res., 44:317 (1995), Davis et al., Biochem., 35:1270 (1996)). Thus it has been widely accepted in the art that MCTA acts only as a prodrug; i.e., a compound which is inactive in and of itself but is biochemically converted to an active specie in vivo.

Contrary to the preceding accepted theory that MCTA is but a prodrug, we have found that, when MCTA is administered in such a manner that biochemical conversion to A771726 is either eliminated or at least drastically curtailed, MCTA itself is an extremely effective modulator of PDGFR and FGFR activity and is an effective chemotherapeutic agent, especially against solid tumor cancers, both alone and in combination with known chemotherapeutic agents such as BCNU. In addition, the novel analogs of MCTA which are described herein demonstrate activity which is similar to that of unmetabolized MCTA and, like unmetabolized MCTA, is different from that of A771726. That is, the ability of A771726 to inhibit pyrimidine biosynthesis is overcome by the addition of uridine which is characteristic of pyrimide biosynthesis inhibitors. On the other hand, the compounds of the present invention, while structurally similar to MCTA, are capable of inhibiting cellular growth by a mechanism not affected by the addition of

uridine. While not being bound to any particular theory, applicants believe that is due either to the fact that the heteroaryl group of the claimed compounds do not metabolize to an open form at all and therefore exhibit their activity in their native configuation, or, if they do metabolize to an open form, the chemical composition of the open form to which they are converted is either inactive or active, but not as an inhibitor of pyrimidine biosynthesis (as evinced by the fact that uridine addition has no effect).

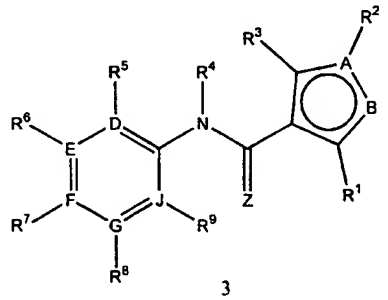
Thus, it appears that, while chemically similar to MCTA, the compounds of the present invention comprise a new family of compounds capable of modulating protein tyrosine kinase activity. While, again, not being bound to a particular theory, it appears that the compounds of this invention affect cell proliferation by modulating PTK signaling. The signaling related to the PTKs, FGFR and PDGFR, appear to be particularly susceptible to modulation by the compounds of the present invention.

As used herein, the terms "modulate", "modulation" or "modulating" refer to the alteration of the catalytic activity of PTKs. In particular, modulating refers to the activation of the catalytic activity of PTKs, more preferably the activation or inhibition of the catalytic activity of PTKs, depending on the concentration of the compound administered or, more preferably still, the inhibition of the catalytic activity of PTKs. Modulation may be effected by direct interaction with a PTK or through intervention at some other point in the biochemical process controlled by the particular PTK, the observable result of which appears as a modulation of PTK catalytic activity.

The term "catalytic activity" as used herein refers to the rate of phosphorylation of tyrosine under the influence, direct or indirect of PTKs.

A. General Structural Features

Thus, in one aspect, the present invention relates to heteroarylcarboxamide compounds having the chemical structure shown in Formula 3:



3

A is selected from the group consisting of oxygen, nitrogen and sulfur.

B is selected from the group consisting of nitrogen and sulfur and it is understood that when B is sulfur and A is nitrogen, the nitrogen is participating in both a single bond and a double bond within the ring so that it cannot be bonded to any atom outside the ring; that is, when B is sulfur, R^2 cannot exist.

D, E, F, G, and J are independently selected from the group consisting of carbon and nitrogen such that the six-member monocylic heteroaryl ring formed is one known in the chemical arts and, furthermore, when D, E, F, G or J is nitrogen, R^5, R^6, R^7, R^8 or R^9, respectively, does not exist.

R^1 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl and heteroalicyclic.

R^2 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, heteroalicyclic, carbonyl, C-carboxy, S-sulfonamido, sulfonyl, hydroxy, alkoxy, trihalomethanesulfonyl, halo, guanyl, C-amido and C-thioamido.

R^3 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl and heteroalicyclic.

Z is selected from the group consisting of oxygen and sulfur.

R^4 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl heteroaryl, heteroalicyclic, sulfonyl, trihalomethanesulfonyl, hydroxy, alkoxy and C-carboxy.

R^5, R^6, R^7, R^8 and R^9 are independently selected from the group consisting of hydrogen, alkyl, trihaloalkyl, alkenyl, alkynyl, cycycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, cycloalkoxy, aryloxy, heteroaryloxy, heteroalicycloxy, thiohydroxy, thioalkyoxy, thiocycloalkoxy, thioheteraryloxy, thioheteralicycloxy, halo, nitro, cyano, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, silyl, phosphonyl, C-carboxy, O-carboxy, N-amido, C-amido, sulfinyl, sulfonyl, S-sulfonamido, N-sulfonamido, trihalomethanesulfonyl, guanyl, guanidino, trihalomethanesulfonamido, amino and $-NR^{13}R^{14}$.

R^{13} and R^{14} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, C-carboxy, sulfonyl, trihalomethanesulfonyl and, combined, a five- or six-member heteroalicyclic ring containing at least one nitrogen.

As used herein, the term "alkyl" refers to a saturated aliphatic hydrocarbon including straight chain and branched chain groups. Preferably, the alkyl group has 1 to 20 carbon atoms (whenever a numerical range; e.g. "1-20", is stated herein, it means that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc. up to and including 20 carbon atoms). More preferably, it is a medium size alkyl having 1 to 10 carbon atoms. Most preferably, it is a lower alkyl having 1 to 4 carbon atoms. The alkyl group may be substituted or unsubstituted. When

substituted, the substituent group(s) is preferably one or more individually selected from trihaloalkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, heteroaryloxy, heteroalicylcoxy, thiohydroxy, thioalkoxy, thioaryloxy, thioheteroaryloxy, thioheteroalicyloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-carboxy, O-carboxy, nitro, sulfinyl, sulfonyl, sulfonamido, trihalomethanesulfonamido, trihalomethanesulfonyl, silyl, guanyl, guanidino, ureido, phosphonyl, amino and $NR^{13}R^{14}$.

R^{13} and R^{14} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, C-carboxy, sulfonyl, trihalomethysulfonyl and, combined, a five- or six-member heteroalicyclic ring.

A "cycloalkyl" group refers to an all-carbon monocyclic or fused ring (i.e., rings which share an adjacent pair of carbon atoms) group wherein one of more of the rings does not have a completely conjugated pi-electron system. Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, cycloheptane and, cycloheptatriene. A cycloalkyl group may be substituted or unsubstituted. When substituted, the substituent group(s) is preferably one or more individually selected from alkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, heteroaryloxy, heteroalicylcoxy, thiohydroxy, thioalkoxy, thioaryloxy, thioheteroaryloxy, thioheteroalicyloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-carboxy, O-carboxy, nitro, sulfinyl, sulfonyl, sulfonamido, trihalomethanesulfonamido, trihalomethanesulfonyl, silyl, guanyl, guanidino, ureido, phosphonyl, amino and $NR^{13}R^{14}$ with R^{13} and R^{14} as defined above.

An "alkenyl" group refers to an alkyl group, as defined herein, consisting of at least two carbon atoms and at least one carbon-carbon double bond.

An "alkynyl" group refers to an alkyl group, as defined herein, consisting of at least two carbon atoms and at least one carbon-carbon triple bond.

An "aryl" group refers to an all-carbon monocyclic or fused-ring polycyclic (i.e., rings which share adjacent pairs of carbon atoms) groups having a completely conjugated pi-electron system. Examples, without limitation, of aryl groups are phenyl, naphthalenyl and anthracenyl. The aryl group may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more selected from alkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, heteroaryloxy, heteroalicylcoxy, thiohydroxy, thioalkoxy, thioaryloxy, thioheteroaryloxy, thioheteroalicyloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-carboxy, O-carboxy, nitro, sulfinyl, sulfonyl, sulfonamido, trihalomethanesulfonamido, trihalomethanesulfonyl, silyl, guanyl, guanidino, ureido, phosphonyl, amino and $NR^{13}R^{14}$ with R^{13} and R^{14} as defined above.

As used herein, a "heteroaryl" group refers to a monocyclic or fused ring (i.e., rings which share an adjacent pair of atoms) group having in the ring(s) one or more atoms selected from the group consisting of nitrogen, oxygen and sulfur and, in addition, having a completely conjugated pi-electron system. Examples, without limitation, of heteroaryl groups are pyrrole, furan, thiophene, imidazole, oxazole, thiazole, pyrazole, pyridine, pyrimidine, quinoline, isoquinoline, purine and carbazole. The heteroaryl group may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more selected from alkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, heteroaryloxy, heteroalicylcoxy, thiohydroxy, thioalkoxy, thioaryloxy, thioheteroaryloxy, thioheteroalicyloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-

carboxy, O-carboxy, nitro, sulfinyl, sulfonyl, sulfonamido, trihalomethanesulfonamido, trihalomethanesulfonyl, silyl, guanyl, guanidino, ureido, phosphonyl, amino and $NR^{13}R^{14}$ with R^{13} and R^{14} as defined above.

A "heteroalicyclic" group refers to a monocyclic or fused ring group having in the ring(s) one or more atoms selected from the group consisting of nitrogen, oxygen and sulfur. The rings may also have one or more double bonds. However, the rings do not have a completely conjugated pi-electron system. The heteroalicyclic ring may be substituted or unsubstituted. When substituted, the substituted group(s) is preferably one or more selected from alkyl, cycloalkyl, aryl, heteroaryl, heteroalicyclic, hydroxy, alkoxy, aryloxy, heteroaryloxy, heteroalicylcoxy, thiohydroxy, thioalkoxy, thioaryloxy, thioheteroaryloxy, thioheteroalicyloxy, cyano, halo, carbonyl, thiocarbonyl, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, C-amido, N-amido, C-carboxy, O-carboxy, nitro, sulfinyl, sulfonyl, sulfonamido, trihalomethanesulfonamido, trihalomethanesulfonyl, silyl, guanyl, guanidino, ureido, phosphonyl, amino and $NR^{13}R^{14}$ with R^{13} and R^{14} as defined above.

A "hydroxy" group refers to an -OH group.

An "alkoxy" group refers to both an -O-alkyl and an -O-cycloalkyl group, as defined herein.

An "aryloxy" group refers to both an -O-aryl and an -O-heteroaryl group, as defined herein.

A "heteroaryloxy" group refers to a heteroaryl-O- group with heteroaryl as defined herein.

A "heteroalicycloxy" group refers to a heteroalicyclic-O- group with heteroalicyclic as defined herein.

A "thiohydroxy" group refers to an -SH group.

A "thioalkoxy" group refers to both an S-alkyl and an -S-cycloalkyl group, as defined herein.

A "thioaryloxy" group refers to both an -S-aryl and an -S-heteroaryl group, as defined herein.

A "thioheteroaryloxy" group refers to a heteroaryl-S- group with heteroaryl as defined herein.

A "thioheteroalicycloxy" group refers to a heteroalicyclic-S- group with heteroalicyclic as defined herein.

A "carbonyl" group refers to a -C(=O)-R" group, where R" is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, heteroaryl (bonded through a ring carbon) and heteroalicyclic (bonded through a ring carbon), as each is defined herein.

An "aldehyde" group refers to a carbonyl group where R" is hydrogen.

A "thiocarbonyl" group refers to a -C(=S)-R" group, with R" as defined herein.

A "trihalomethanecarbonyl" group refers to a $X_3CC(=O)$- group with X as defined herein.

A "C-carboxy" group refers to a -C(=O)O-R" groups, with R" as defined herein.

An "O-carboxy" group refers to a R"C(=O)O- group, with R" as defined herein.

A "carboxylic acid" group refers to a C-carboxyl group in which R" is hydrogen.

A "halo" group refers to fluorine, chlorine, bromine or iodine.

A "trihalomethyl" group refers to a $-CX_3$ group wherein X is a halo group as defined herein.

A "trihalomethanesulfonyl" group refers to a $X_3CS(=O)_2$- groups with X as defined above.

A "trihalomethanesulfonamido" group refers to a $X_3CS(=O)_2NR^{13}$- group with X and R^{13} as defined herein.

A "cyano" group refers to a -C≡N group.

A "sulfinyl" group refers to a -S(=O)-R" group, with R" as defined herein.

A "sulfonyl" group refers to a $-S(=O)_2R$" group, with R" as defined herein.

An "S-sulfonamido" group refers to a $-S(=O)_2NR^{13}R^{14}$, with R^{13} and R^{14} as defined herein.

An "N-Sulfonamido" group refers to a $R^{13}S(=O)_2NR^{14}-$ group, with R^{13} and R^{14} as defined herein.

An "O-carbamyl" group refers to a $-OC(=O)NR^{13}R^{14}$ group with R^{13} and R^{14} as defined herein.

An "N-carbamyl" group refers to a $R^{13}OC(=O)NR^{14}$ group, with R^{13} and R^{14} as defined herein.

An "O-thiocarbamyl" group refers to a $-OC(=S)NR^{13}R^{14}$ group with R^{13} and R^{14} as defined herein.

An "N-thiocarbamyl" group refers to a $R^{13}OC(=S)NR^{14}-$ group, with R^{13} and R^{14} as defined herein.

An "amino" group refers to an $-NR^{13}R^{14}$ group in which both R^{13} and R^{14} are hydrogen.

A "C-amido" group refers to a $-C(=O)NR^{13}R^{14}$ group with R^{13} and R^{14} as defined herein.

An "N-amido" group refers to a $R^{13}C(=O)NR^{14}-$ group, with R^{13} and R^{14} as defined herein.

A "C-thioamido" group refers to a $-C(=S)NR^{13}R^{14}$ group with R^{13} and R^{14} and defined herein.

A "ureidyl" group refers to a $-NR^{12}C(=O)NR^{13}R^{14}$ group, with R^{13} and R^{14} as defined herein and R^{12} defined the same as R^{13} and R^{14}.

A "guanidinyl" group refers to a $-R^{12}NC(=N)NR^{13}R^{14}$ group, with R^{12}, R^{13} and R^{14} as defined herein.

A "guanyl" group refers to a $R^{13}R^{14}NC(=N)-$ group, with R^{13} and R^{14} as defined herein.

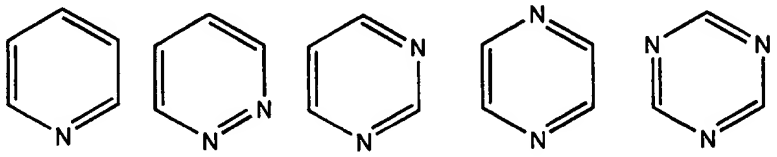
A "nitro" group refers to a $-NO_2$ group.

A "silyl" group refers to a $-Si(R")_3$, with R" as defined herein.

A "methylenedioxy" group refers to a $-OCH_2O-$ group where the two oxygens are covalently bonded to two adjacent carbon atoms of an aryl or heteroaryl ring with aryl and heteroaryl as defined herein.

A "1,3-dioxano" group refers to a $-CH_2OCH_2O-$ group where the $-CH_2$ and the oxygen are covalently bonded to two adjacent carbon atoms of an aryl or heteroaryl ring with aryl and heteroaryl as defined herein.

Examples of six-member monocyclic heteroaryl rings known in the chemical arts include, but are not limited to the following:



B. Preferred Structural Features.

Preferred structural features for the claimed compounds are those in which:

A is oxygen and B is nitrogen;

R^1 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl and alkynyl; and,

R^3 is selected from the group consisting of hydrogen, alkyl, cycloalkyl and aryl; and,

R^4 is hydrogen.

Further preferred structures for the claimed compounds are those in which:

A and B are nitrogen;

R^2 is selected from the group consisting of hydrogen, alkyl and cycloalkyl;

Z is oxygen;

R^5, R^8 and R^9 are hydrogen;

R^6 is selected from the group consisting or hydrogen and alkyl; and,

R^7 is selected from the group consisting of hydrogen, trihalomethyl and trihalomethanesulfonyl.

Other preferred embodiments of the present invention are those in which:

R^6 and R^7, combined, form a methylenedioxy or a 1,3-dioxano group.

And, finally, a preferred structure for the claimed compounds is that in which J is nitrogen.

The Biochemistry

A. Modulation of Protein Kinase Catalytic Activity Generally

In another aspect, this invention relates to a method for the treatment or prevention of a disorder characterized by inappropriate PK activity comprising administering to a patient afflicted with such a disorder a therapeutically effective amount of one or more of the disclosed compounds, or a physiologically acceptable salt thereof, alone or in a pharmaceutically acceptable composition.

The term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by, practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

As used herein, the terms "prevent", "preventing" and "prevention" refer to a method for barring an organism from acquiring a PTK mediated cellular disorder in the first place.

As used herein, the terms "treat", "treating" and "treatment" refer to a method of alleviating or abrogating a PTK mediated cellular disorder and/or its attendant symptoms. With regard particularly to cancer, these terms simply mean that the life expectancy or an individual afflicted with a cancer will be increased or that one or more of the symptoms of the disease will be reduced.

A "disorder characterized by inappropriate PTK activity" includes, but is not limited to, cell proliferative disorders, cell differentiation disorders, cell growth disorders and

metastatic disorders. Such disorders include, but are not limited to, cancer, as described below and, in addition, the development of neoplasia such as carcinoma, sarcoma, glioblastoma and hemangioma, leukemia, psoriasis, arteriosclerosis, arthritis and diabetic retinopathy (or other disorders related to uncontrolled angiogenesis and/or vasculogenesis), fibrotic disorders and metabolic disorders.

As used herein, the term "cancer" refers to various types of malignant neoplasms, most of which can invade surrounding tissues, and may metastasize to different sites, as defined by Stedman's Medical Dictionary 25th edition (Hensyl ed. 1990). Examples of cancers which may be treated by the present invention include, but are not limited to, brain, ovarian, colon, prostate, kidney, bladder, breast, lung, oral and skin cancers which exhibit inappropriate PTK activity. These types of cancers can be further characterized. For example, brain cancers include glioblastoma multiforme, anaplastic astrocytoma, astrocytoma, ependymoma, oligodendroglioma, medulloblastoma, meningioma, sarcoma, hemangioblastoma, and pineal parenchymal. Skin cancers include melanoma and Kaposi's sarcoma.

Unwanted cell proliferation can result from inappropriate PTK activity occurring in different types of cells including cancer cells, cells surrounding a cancer cell (stromal cells), endothelial cells and smooth muscle cells. For example, and without limitation, an increase in FGFR and/or PDGFR activity of endothelial cells surrounding cancer cells may lead to an increased vascularization (angiogenesis) of the tumor, thereby facilitating growth of the cancer cells. Inappropriate PTK activity may also contribute to the proliferation of cancer cells by direct mitogenic stimulation.

"Cell proliferative disorders" refer to disorders wherein unwanted cell proliferation of one or more subset of cells in a multicellular organism occurs resulting in harm (e.g., discomfort or decreased life expectancy) to the multicellular

organism. Cell proliferative disorders can occur in different types of animals and in humans. Cell proliferative disorders include cancers, skeletal disorders, angiogenic or blood vessel proliferative disorders, fibrotic disorders and mesangial cell proliferative disorders.

Blood vessel proliferative disorders refer to angiogenic and vasculogenic disorders generally resulting in abnormal proliferation of blood vessels. The formation and spreading of blood vessels, or vasculogenesis and angiogenesis, respectively, play important roles in a variety of physiological processes such as embryonic development, corpus luteum formation, wound healing and organ regeneration. However, they also play a pivotal role in cancer development. Other examples of blood vessel proliferation disorders include arthritis, where new capillary blood vessels invade the joint and destroy cartilage, and ocular diseases, like diabetic retinopathy, where new capillaries in the retina invade the vitreous, bleed and cause blindness. Conversely, disorders related to the shrinkage, contraction or closing of blood vessels, such as restenosis, are also implicated.

Fibrotic disorders refer to the abnormal formation of extracellular matrices. Examples of fibrotic disorders include hepatic cirrhosis and mesangial cell proliferative disorders. Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. An increased extracellular matrix resulting in a hepatic scar can also be caused by viral infection such as hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis. Other fibrotic disorders implicated include atherosclerosis.

Mesangial cell proliferative disorders refer to disorders brought about by abnormal proliferation of mesangial cells. Mesangial proliferative disorders include various human renal diseases, such as glomerulonephritis, diabetic nephropathy and malignant nephrosclerosis as well as disorders such as

thrombotic microangiopathy syndromes, transplant rejection, and glomerulopathies. PDGF-R has been implicated in the maintenance of mesangial cell proliferation. Floege et al., 1993, Kidney International 43:47S-54S.

Other examples of cell proliferative disorders are disclosed in the following references which are incorporated as if fully set forth herein. The EGFR (Tuzi et al., 1991, Br. J. Cancer 63:227-233; Torp et al., 1992, APMIS 100:714-719) HER2/neu (Slamon et al., 1989, Science 244:707-712) and the PDGF-R (Kumabe et al., 1992, Oncogene, 7:627-633) are over-expressed in many tumors and/or persistently activated by autocrine loops. In fact, in the most common and severe cancers these receptor over-expressions (Akbasak and Suner-Akbasak et al., 1992, J. Neurol. Sci., 111:119-143; Dickson et al., 1992, Cancer Treatment Res. 61:249-273; Korc et al., 1992, J. Clin. Invest. 90:1452-1460) and autocrine loops (Lee and Donoghue, 1992, J. Cell. Biol., 118:1057-1070; Korc et al., supra; Akbasak and Suner-Akbasak et al., supra) have been demonstrated. For example, EGFR has been associated with squamous cell carcinoma, astrocytoma, glioblastoma, head and neck cancer, lung cancer and bladder cancer. HER2 has been associated with breast, ovarian, gastric, lung, pancreatic and bladder cancer. PDGFR has been associated with glioblastoma, lung cancer, ovarian cancer, prostate cancer, and melanoma. The RTK c-met has been associated with hepatocarcinogenesis. Additionally, c-met has been linked to other malignant tumor formations. More specifically, c-met has been associated with, among other cancers, colorectal, thyroid, pancreatic and gastric carcinoma, leukemia and lymphoma. Additionally, over-expression of the c-met gene has been detected in patients with Hodgkins disease and Burkitts disease.

IGF-IR, in addition to being implicated in nutritional support and in type-II diabetes, has also been associated with several types of cancers. For example, IGF-I has been implicated as an autocrine growth stimulator for several tumor

types, e.g. human breast cancer carcinoma cells (Arteaga et al., 1989, J. Clin. Invest. 84:1418-1423) and small lung tumor cells (Macauley et al., 1990, Cancer Res., 50:2511-2517). In addition, IGF-I, while integrally involved in the normal growth and differentiation of the nervous system, also appears to be an autocrine stimulator of human gliomas. Sandberg-Nordqvist et al., 1993, Cancer Res. 53:2475-2478. The importance of the IGF-IR and its ligands in cell proliferation is further supported by the fact that many cell types in culture (fibroblasts, epithelial cells, smooth muscle cells, T-lymphocytes, myeloid cells, chondrocytes, osteoblasts (the stem cells of the bone marrow)) are stimulated to grow by IGF-I. Goldring and Goldring, 1991, Eukaryotic Gene Expression,1:301-326. In a series of recent publications, Baserga suggests that IGF-IR plays a central role in the mechanisms of transformation and, as such, could be a preferred target for therapeutic interventions for a broad spectrum of human malignancies. Baserga, 1995, Cancer Res., 55:249-252; Baserga, 1994, Cell 79:927-930; Coppola et al., 1994, Mol. Cell. Biol., 14:4588-4595.

The term "organism" refers to any living entity comprised of at least one cell. A living organism can be as simple as, for example, a single eukariotic cell or as complex as a mammal, including a human being.

The term "therapeutically effective amount" as used herein refers to that amount of the compound being administered which will relieve to some extent one or more of the symptoms of the disorder being treated. In reference to the treatment of cancer, a therapeutically effective amount refers to that amount which has the effect of (1) reducing the size of the tumor; (2) inhibiting (that is, slowing to some extent, preferably stopping) tumor metastasis; (3) inhibiting to some extent (that is slowing to some extent, preferably stopping) tumor growth; and/or, (4) relieving to some extent (or

preferably eliminating) one or more symptoms associated with the cancer.

A "therapeutically effective amount", in reference to the treatment of a cell proliferative disorder other than a cancer refers to an amount sufficient to bring about one or more of the following results: inhibit the growth of cells causing the disorder, relieve discomfort due to the disorder, or prolong the life of a patient suffering from the disorder.

The association between abnormal PTK activity and disease is not restricted to cancer. For example, RTKs have been associated with metabolic diseases like psoriasis, diabetes mellitus, wound healing, inflammation, and neurodegenerative diseases. For example, EGF-R is indicated in corneal and dermal wound healing. Defects in the Insulin-R and the IGF-1R are indicated in type-II diabetes mellitus. A more complete correlation between specific RTKs and their therapeutic indications is set forth in Plowman et al., DN&P 7:334-339 (1994).

As noted previously, not only RTKs but CTKs as well including, but not limited to, src, abl, fps, yes, fyn, lyn, lck, blk, hck, fgr and yrk (reviewed by Bolen et al., FASEB J., 6:3403-3409 (1992)) are involved in the proliferative and metabolic signal transduction pathway and thus were expected, and have been shown, to be involved in many PTK-mediated disorders to which the present invention is directed. For example, mutated src (v-src) has been demonstrated as an oncoprotein ($pp60^{v-src}$) in chicken. Moreover, its cellular homolog, the proto-oncogene $pp60^{c-src}$ transmits oncogenic signals of many receptors. For example, over-expression of EGFR or HER2/neu in tumors leads to the constitutive activation of $pp60^{c-src}$, which is characteristic of malignant cells but absent from normal cells. On the other hand, mice deficient in the expression of c-src exhibit an osteopetrotic phenotype, indicating a key participation of c-src in osteoclast function

and a possible involvement in related disorders. Similarly, Zap70 is implicated in T-cell signaling.

B. Modulation of FGFR and PDGFR Catalytic Activity

A further feature of this invention is a method for treating a disorder characterized by inappropriate FGFR activity comprising administering MCTA in a pharmaceutical composition to a patient in need of such treatment. In particular the disorder comprises cancer.

Still another feature of this invention is a method for treating a disorder characterized by inappropriate PDGFR activity likewise comprising administering MCTA or a compound of this invention in a pharmaceutical composition to a patient in need of such treatment. In particular, the disorder comprises cancer.

Unwanted cell proliferation can result from inappropriate FGFR activity occurring in different types of cells including cancer cells, cells surrounding a cancer cell (stromal cells), endothelial cells and smooth muscle cells. For example, an increase in FGFR activity of endothelial cells surrounding cancer cells may lead to an increased vascularization (angiogenesis) of the tumor, thereby facilitating growth of the cancer cells. Inappropriate FGFR activity may also contribute to the proliferation of cancer cells by direct mitogenic stimulation.

"Inappropriate FGFR activity" refers to either 1) FGFR expression in cells which normally do not express FGFR; 2) FGF ligand expression by cells which normally do not express FGF; 3) increased FGFR expression leading to a disorder; 4) increased FGF expression leading to a disorder; or 5) mutations leading to constitutive activation of FGFR. The existence of inappropriate or abnormal FGF and FGFR levels or activities is determined by procedures well known in the art and are discussed in greater detail below.

Inappropriate FGFR activity can also result in metastatic disorders. Metastasis is the term used to describe the process by which a primary tumor spreads to distant sites in the body, forming secondary tumors. Metastatic disease is often more deadly than a primary cancer. The process and consequences of metastasis are described more fully below.

The methods of the invention are designed to inhibit unwanted cell proliferation or metastasis by altering the activity of FGFR. Without being bound to any theory, inhibition of the activity of FGFR may occur by inhibiting tyrosine phosphorylation of FGFR, by inhibiting substrate or adaptor protein binding to the receptor, or by inhibiting other downstream signaling events, thereby inhibiting the activity of FGFR. However, unless otherwise stated, the use of the claimed methods and compositions are not limited to this particular theory. When used herein the term "compositions" refers to pharmaceutical compositions of MCTA or any of the other compounds of this invention useful in the claimed method.

The compositions can be used to treat a disorder characterized by inappropriate FGFR activity by administering a therapeutically effective amount of the composition to a patient (i.e. a human or an animal) having a disorder. MCTA and the other heteroarylcarboxamides of this invention may also be used in in vitro studies of the mechanism of action of FGFR or FGF itself.

"Significant" inhibition of receptor tyrosine kinase activity refers to an IC50 of less than or equal to 100 μM using one or more of the assays described in the Examples infra. Preferably, the compound can inhibit FGFR activity with an IC50 of less than or equal to 85 μM. A lower IC50 is preferred because the IC50 provides an indication of the in vivo effectiveness of the compound. Other factors known in art, such as compound half-life, biodistribution, and toxicity should also be considered for therapeutic uses. Such factors may enable a compound with a higher IC50 to have greater in

vivo efficacy than a compound having a lower IC50. The term "significant" can also refer to comparisons between tumor growth in animals treated with a compound of the invention and a control animal wherein the reduction in growth is found to be statistically different.

Another useful feature of MCTA and the compounds of this invention is receptor specificity. This is not to say that the compounds will have absolute specificity, however they show selective inhibition of a limited number of RTKs and, therefore, will be useful only in disorders characterized by inappropriate activity of that limited set of RTKs, i.e. FGFR. Selective inhibition of the FGFR family is achieved by significantly inhibiting FGFR activity, while having an insignificant effect on PTK activity from a disparate family (e.g., an IC50 greater than 100 μM on EGFR).

The downstream signaling cascade of events from ligand binding to biological response has not been fully elucidated for any RTK. The intracellular environment is highly complex. While it can be demonstrated that particular signaling molecules play roles in many pathways, it cannot be assumed that signaling from any two RTKs is the same. It is known in the art, for example, that two highly related RTKS, EGFR and Her2, signal, at least in part, through different pathways. (See Fazioli, et al., J. Bio. Chem. 267(8):5155, 1992; Di Fiore, et al., Cell 51:1063, 1987; Segatto, et al., Mol. Cell. Bio. 11:3191, 1991; and Dougall, et al., Oncogene 9:2109, 1994.) Thus, although it has been previously demonstrated that MCTA inhibits signaling by PDGFR (See WO 95/19169, published July 20, 1995) it would not be assumed that it would also be effective at inhibiting the activity of FGFR, which belongs to a distinct family of receptors. And, of course, it follows that MCTA would therefore not be expected to be useful in treating a distinct set of disorders, i.e., those characterized by inappropriate FGFR activity.

MCTA, as is the case with the other compounds of this invention, is preferably administered in a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is a formulation to which the compound can be added to dissolve or otherwise facilitate administration of the compound. Examples of pharmaceutically acceptable carriers include water, saline, physiologically buffered saline, cyclodextrins and VPD:D5W (discussed further below). Hydrophobic compounds such as MCTA are preferably administered using a carrier such as VPD:D5W. An important factor in choosing an appropriate pharmaceutically acceptable carrier is choosing a carrier in which the compound remains active or the combination of the carrier and the compound produces an active compound. The compound may also be administered in a continuous fashion using a slow release formulation or a pump to maintain a constant or varying drug level in a patient.

As discussed above, the FGFR family contains at least four distinct members: FGFR1 (also called Flg and Cek1), FGFR2 (also called Bek, Ksam, KsamI and Cek3), FGFR3 (also called Cek2) and FGFR4. They share a common structure consisting of, in the mature protein, one or more immunoglobulin-like (IgG-like) loops flanked by characteristic cysteines, a hydrophobic transmembrane domain and a intracellular domain containing a catalytic region that is split by a short insert. (See Ullrich and Schlessinger, Cell 61:203, 1990.) The degree of homology varies between them, with the highest homology found in the catalytic domain. Additional diversity in the family is created through splice variants that vary the number and character of the IgG-like regions in the extracellular domain. At least nine FGFR ligands have been identified including FGF1 (acidic FGF), FGF2 (basic FGF), FGF3 (int-2), FGF4 (Kaposi FGF), FGF5, FGF6, FGF7 (keratinocyte growth factor (KGF)), FGF8 (androgen-induced growth factor) and FGF9.

Multiple members of the FGF ligand family can bind to the same receptor species. For a general review of FGFs and FGFRs see Johnson and Williams, Adv. in Cancer Res. 60:1, 1993.

The PDGF receptor family contains only the two isoforms PDGFR-α and PDGFR-ß, which are known to heterodimerize. PDGF ligand is a pleiotropic factor that exists as a homo- or heterodimer of two polypeptides, the A- and B- chains (Habenicht, et al, Klin. Wochen-Schrift 68:53, 1990; Heldin, EMBO J. 11:4251, 1992).

Other tyrosine kinase receptors structurally and functionally related to FGFR and PDGFR include Flt (de Vries, et al, Science 255:989, 1992) and KDR (Terman, et al, BBRC 187;1579, 1992), both of which are activated by the ligand VEGF (Rosenthal, et al., Growth Factors, 4:53-59, 1990; Conn, et al., Proc. Natl. Acad. Sci. (USA), 87:1423-1427, 1990; Houck, et al., Mol. Endocrinol., 5:1806-1814, 1991). VEGF expression is known to be increased by hypoxia (such as would be found in growing tumors) and is known to stimulate endothelial cells and to be involved in angiogenesis (Plate et al., Nature, 359:845-848, 1992; Shweike, et al., Nature 359:843-845, 1992).

PDGF- and FGF-dependent signaling is initiated immediately following binding of ligand to a receptor. Ligand binding induces receptor dimerization, either homodimers or heterodimers, leading to activation of receptor tyrosine kinase activity and autophosphorylation. Activation of the receptor leads to increased tyrosine phosphorylation on a number of cellular proteins, although many of their identities and functions are still largely unknown. Depending on the cell type, PDGFR and/or FGFR activation ultimately leads to proliferation, differentiation, inhibition of differentiation, motility, etc.

The use of the present invention is facilitated by first determining whether a disorder is related to inappropriate PDGFR and/or FGFR activity. Once such disorders are identified as such, patients suffering from the disorder can be identified

by analysis of their symptoms by procedures well known in the medical arts. Such patients can then be treated as described herein. Many well known techniques exist for determining whether a disorder is related to inappropriate PDGFR and/or FGFR activity. For example, comparisons can be made in the level of expression of either FGF and/or PDGF ligand or FGFR and/or PDGFR, in a tumor biopsy with levels in similar normal tissues or tumor cells known to be unrelated to FGFR and/or PDGFR activity (such as A431 cells, Yaish, et al, Science 242:933, 1988). Such comparisons can be done by immunostaining with FGFR and/or PDGFR specific antibodies or binding and detection of FGF and/or PDGF ligand using techniques well known in the art, by Northern blot analysis for the presence of ligand or receptor RNA, or by transcript imaging (Plowman, WO96/34985, published 7 November 1996, and incorporated by reference herein). Alternatively samples can be analyzed for level of receptor phosphorylation, which is indicative of activity, compared to normal tissues. Receptor phosphorylation is readily detected by means well known in the art such as by using anti-phosphotyrosine antibodies. If the cancer cells have a higher level of FGFR and/or PDGFR activity or expression than non-FGFR and/or PDGFR driven cancers or normal tissues, preferably equal to or greater than previously identified FGFR and/or PDGFR driven cancers, then they are candidates for treatment using the described inhibitors.

In the case of cell proliferative disorders arising due to unwanted proliferation of non-cancer cells, the level of receptor activity is compared to that level occurring in the general population (e.g., the average level occurring in the general population of people or animals excluding those people or animals suffering from a cell proliferative disorder). If the unwanted cell proliferation disorder is characterized by a higher receptor level then occurring in the general population then the disorder is a candidate for treatment using the described inhibitors.

C. FGFR- and PDGFR-related Disorders

One class of PTK disorders which involve FGFR and/or PDGFR are cell proliferative disorders. As discussed above, all proliferative disorders result in unwanted cell proliferation of one or more subsets of cells in a multicellular organism resulting in harm to the organism. Two ways in which inappropriate PTK/FGFR/PDGFR activity can stimulate unwanted cell proliferation of a particular type of cell are by directly stimulating growth of the particular cell, or by increasing vascularization of a particular area (angiogenesis), such as tumor tissue, thereby facilitating growth of the tissue. Angiogenesis also plays a significant role in metastasis, a complex disorder which is discussed in more detail below.

Cell proliferative disorders include cancers, blood vessel proliferation disorders, skeletal malformations and fibrotic disorders. These disorders are not necessarily independent. For example, fibrotic disorders may be related to, or overlap with, blood vessel disorders. For example, Moyamoya disease (which is characterized herein as a blood vessel disorder) results in the abnormal formation of fibrous tissue in the intracranial arteries.

Not all cancers found in a particular location within the body will be treatable by the method of the invention, only those characterized by inappropriate FGFR, PDGFR or related receptor activity. For example, only 30% of bladder carcinomas are characterized as highly invasive and prone to metastasis (Raghavan, et al, NEJM 322:1129, 1990; Allen and Maher, J. Cell. Physiol. 155:368, 1993), and these have been associated with FGFR activity (Allen and Maher, supra). Breast cancers are associated with inappropriate FGFR activity in 12% - 32% of cases (Adnane, et al, Oncogene 6:659, 1991; Penault-Llorca, et al, Int. J. Cancer 61:170, 1995). Friess, et al., (Chirug 65:604, 1994) report that approximately 50% of primary pancreatic cancers surveyed express FGFR, and expression was

associated with tumor aggressiveness as measured by significantly shorter post-operative survival. Holm, et al. (Int. J. Oncology 9:1077, 1996) found expression of PDGFR in only 30% of non-small cell lung cancer cell lines examined, and only 10% expressed KDR. Seymour, et al (supra) found expression of PDGF in 43% of tumors from breast cancer patients. Expression of PDGF correlated with a reduced chance of survival. One can determine which cancers are treatable by the compounds and methods of the invention by employing the techniques described above for determining inappropriate FGFR and/or PDGFR activity.

The formation and spreading of blood vessels, or vasculogenesis and angiogenesis respectively, play important roles in a variety of physiological processes such as embryonic development, wound healing and organ regeneration. They also play a role in cancer development. Blood vessel proliferation disorders refer to angiogenic and vasculogenic disorders generally resulting in abnormal proliferation of blood vessels. Examples of such disorders besides cancer include Moyamoya disease and macular degeneration. FGFR and KDR have been recognized has having a regulatory role in angiogensis, along with other factors, due to their role in both endothelial cell proliferation and migration (Friesel and Maciag, FASEB J. 9:919, 1995; Folkman and Klagsbrun, Science 235:442, 1987; Mullins and Rifkin, J. Cell. Physiol. 119:247, 1984; Gualandris, et al., Cell Growth & Diff. 7:147, 1996). FGFR activity has been suggested to play a role specifically in the angiogenesis associated with macular degeneration (Amin, et al, Invest. Ophthal. and Vis. Sci. 35(8):3178, 1994). PDGF and VEGF expression is associated with angiogeneis and metastasis in breast cancer (Anan, et al., Surgery:119:333, 1996). PDGF expression has been correlated with increased blood vessel count in colon cancers (Hsu, et al, J. Cell. Physol. 165:239, 1995). PDGF and FGF have been shown to induce secretion of VEGF by glioma cells (Tsai, et al, J. Neurosurg. 82:864, 1995).

Moyamoya disease is characterized by intracranial carotid artery stenosis and occlusions and a fine network of vessels at the base of the brain and may be described as both an angiogenic and fibrotic disorder (Suzuki and Kodama, Stroke 14:104, 1983; Suzuki and Takaku, Arch Neurol 20:288, 1969). Suzui, et al, (Neurosurgery 35(1):20, 1994) have found that both FGF ligand and FGFR are increased in the superficial temporal artery of patients with Moyamoya disease.

Fibrotic disorders refer to the abnormal formation of extracellular matrix. Examples of fibrotic disorders include those found in the liver (hepatic cirrhosis), kidney (glomerular sclerosis, interstitial nephritis), lung (interstitial pulmonary fibrosis), arteries (restenosis, atherosclerosis) and skin (wound scarring, scleroderma).

Hepatic cirrhosis is characterized by the increase in extracellular matrix constituents resulting in the formation of a hepatic scar. An increased extracellular matrix resulting in a hepatic scar can also be caused by viral infection such as hepatitis. Lipocytes appear to play a major role in hepatic cirrhosis. Inappropriate FGFR activity can stimulate lipocyte proliferation.

As noted above, other proliferative disorders involving FGFR, PDGFR and other PTKs can be identified by standard techniques, and by determination of the efficacy of action of the compound described herein.

D. Metastatic Disorders

Liotta, et al describe invasion and metastasis as "the most life-threatening aspects of the oncogenic process" (See review in Liotta, et al, Cell 64:327, 1991). Invasion and metastasis are complex events mediated by a group of coordinated cellular processes. They are facilitated by proteins that stimulate tumor cell attachment to extracellular matrix, proteolysis of barriers such as the basement membrane, migration into the circulatory system and attachment to and

colony formation in distant organs. There is also a significant correlation between metastatic potential and angiogeneic potential, and the two processes may have many factors in common. (See Claffey, et al, Cancer Res. 56:172, 1996; Takahashi, et al., Cancer Res. 55:3964, 1995.)

The cellular responses induced by FGF, PDGF and VEGF ligands include those necessary for metastasis: proliferation, migration, production of proteases, and neovascularization. FGFR and PDGFR expression has been associated with increased aggressiveness and metastasis of a number of cancers. Nakamoto, et al (Cancer Research 52:571, 1992) compared FGF and FGFR expression and responsiveness in several human prostate cancer cell lines. The degree of metastasis in murine models shown by each of the cell lines LNCaP, DU145 and PC3 correlated directly with FGFR expression (with LNCaP the lowest and PC3 the highest), although the biological consequences of FGFR expression were not studied in depth. Allen and Maher, J. Cell. Phys. 155:368, 1993) report a similar study with bladder carcinoma cell lines. In a comparison of invasive and non-invasive tumors the invasive tumor (EJ) showed a significant increase in FGFR at both the protein and RNA level, with the non-invasive tumor (RT4) showing almost no FGFR present. The FGFR was also shown to be biologically active by receptor phosphorylation in response to ligand. Anan, et al. (supra) found PDGF mRNA was expressed more frequently in breast tumors with lymph node metastases that in those without metastases.

Several groups have shown not only a correlation of invasion/metastasis with FGFR but have demonstrated that inhibition of FGFR activity can inhibit this process. Morrison, et al. (J. Neuro-Oncol. 18:207, 1994) showed a strong association between aggressive gliobastomas and increased expression of FGFRI, while Takahashi, et al. (FEBS 288:65, 1991) demonstrated that a neutralizing antibody against basic FGF ligand was able to inhibit the growth of two human

glioblastomas (U87MG and T98G) that express both FGF ligand and FGFR. Ohata et al. (Br. J. Cancer 72:824, 1995) examined the expression of FGF ligand and FGFR in primary pancreatic carcinomas. A significant correlation was found between FGFR expression level and the presence of retroperitoneal invasion, metastasis to the lymph nodes and tumor stage. High FGFR expression was significantly associated with shorter post-operative survival and extent of malignancy. Hasegawa, et al. (BBRC 200(3):1435, 1994) showed that FGF ligand stimulated basement membrane invasion by human pancreatic cancer cells in an in vitro model compared to other growth factors (EGF, PDGF) and that invasion could be significantly inhibited by the addition of an anti-FGFR antibody.

In addition to the above, both RTKs and CTKs are currently suspected as being involved in hyperimmune disorders.

Thus, in one aspect, this invention is directed to compounds which modulate PTK signal transduction by affecting the enzymatic activity of the PTKs and thereby interfering with the signals transduced by such proteins. More particularly, an aspect of the present invention is directed to compounds which modulate the PTK-mediated signal transduction pathways as a therapeutic approach to treat many kinds of solid tumors, including but not limited to carcinoma, sarcoma, erythroblastoma, glioblastoma, meningioma, astrocytoma, melanoma and myoblastoma. Leukemias may also be susceptible to treatment using the compounds of this invention. Indications may include, but are not limited to brain cancers, bladder cancers, ovarian cancers, gastric cancers, pancreatic cancers, colon cancers, blood cancers, lung cancers, bone cancers and leukemias.

The term "administering" as used herein refers to a method of contacting a compound of this invention with a PTK both in vitro, i.e. in a test tube, and in vivo, i.e. in cells or tissues of a living organism. Thus, the PTK mediated disorders which are the object of this invention can be

studied, prevented or treated by the methods set forth herein whether the cells or tissues of the organism exist within the organism or outside the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. In this context, the ability of a particular compound to affect a PTK related disorder can be determined; i.e., the IC50 of the compound, defined below, before the use of the compounds in more complex living organisms is attempted. For cells outside the organism, multiple methods exist, and are well-known to those skilled in the arts, to administer compounds including, but not limited to, cell micro-injection and numerous carrier techniques. For cells harbored within a living organism, myriad methods also exist, and are likewise well-known to those skilled in the art, to administer compounds including, but not limited to, oral, parenteral, and aerosol applications.

As used herein, "PTK related disorder," "PTK driven disorder", "abnormal PTK activity" and "inappropriate PTK activity" all refer to a disorder characterized by inappropriate or over-activity of PTKs, which can be either RTKs or CTKs. Inappropriate activity refers to either: (1) PTK expression in cells which normally do not express PTKs; (2) increased PTK expression leading to unwanted cell proliferation, differentiation and/or growth; or, (3) decreased PTK expression leading to unwanted reductions in cell proliferation, differentiation and/or growth. Overactivity of PTKs refers to either amplification of the gene encoding a particular PTK or production of a level of PTK activity which can correlate with a cell proliferation, differentiation and/or growth disorder (that is, as the level of the PTK increases, the severity of one or more of the symptoms of the cellular disorder increases).

The methods and compositions of the invention are designed to inhibit unwanted cell proliferation or metastasis by altering the activity of PTKs. Without being bound to any theory, inhibition of the activity of PTKs may occur by

inhibiting tyrosine phosphorylation of a RTK, by inhibiting substrate or adaptor protein binding to the receptor, or by inhibiting other downstream signaling events, thereby inhibiting the activity of the RTK. However, unless otherwise stated, the use of the claimed methods and compositions are not limited to this particular theory.

E. MCTA

MCTA, also known as leflunomide and HWA 486, has been previously discussed in various publications as having a variety of uses including as an antirheumatic, antiphlogistic, antipyretic or analgesic and in the treatment of multiple sclerosis (Kommerer F-J, et al., U.S. Pat. No. 4,284,786 (1981) and Kommerer F-J, et al., U.S. Pat. No. 4,351,841 (1982). It has been suggested as an anti-inflammatory agent (Bartlett, et al., Agents and Actions 32:10-21, 1991; EP 607 775, EP 607 776, EP 607 777). European Application 0 414 329 A2 suggests the use of MCTA for the treatment of ocular disorders associated with an inflammatory response, and (EP 665 014, US 5,519,042) suggests its use in treating vascular disorders.

Hirth, et al., (PCT/US95/00363) assert that MCTA can be used to treat cell proliferative disorders that are related to inappropriate activity of the Platelet Derived Growth Factor Receptor (PDGFR) including PDGFR driven cancer. Bartlett, et al. (PCT/EP90/01800, US 5,532,259 issued July 2, 1996) assert that MCTA can be used to treat cancers, in particular cancers associated with inappropriate activity of the Epidermal Growth Factor Receptor (EGFR). In Bartlett, et al., Agents and Actions 32:10, 1991, the authors state:

> we could show that tyrosine phosphorylation of the RR-SRC peptide substrate and the autophosphorylation of the epidermal growth factor (EGF) receptor were, dose dependently, inhibited by leflunomide.

Mattar et al., FEBS 334:161, 1993 (not admitted to be prior art) describes the use of a metabolite of leflunomide to inhibit EGF-dependent cell growth, including A431 cells. Mattar also asserts that platelet-derived growth factor-dependent tyrosine phosphorylation was also inhibited by A77 1726 in intact cells at concentrations similar to EGF-dependent phosphorylation.

The inventors herein have discovered that the teachings of Bartlett and Mattar may be in error. As illustrated in the examples described below, MCTA inhibits FGFR activity while having little if any effect on EGF-receptor activity in cells. In addition, while MCTA inhibited growth of tumors characterized by inappropriate FGFR activity, it did not significantly inhibit the growth of cells shown in PCT/EP90/01800 (A431 and KB) when tested in in vivo models. This data is surprising in view of the results described by Bartlett et al., supra, (Agents and Actions) in which MCTA was ostensibly shown to inhibit EGF induced EGF receptor autophosphorylation and cell proliferation, and Mattar et al., supra, in which a metabolite of MCTA inhibited the in vitro growth of A431 cells. Furthermore, neither Bartlett or Mattar suggest that MCTA can be used to inhibit metastasis.

The present disclosure demonstrates the ability of MCTA to inhibit the cellular response to FGFR activity and unwanted cell proliferation in vivo, such as that found in cancers characterized by inappropriate FGFR activity. Moreover, the present invention demonstrates that MCTA is useful for inhibiting metastasis.

F. Treatment of Solid Tumor Cancers

In yet another aspect, the present invention relates to a method for inhibiting tumorigenic activity in a cell comprising administering to the cell a combination of drugs including a chemotherapeutic nitrosourea and a cytostatic signal transduction inhibitor. The cytostatic signal

transduction inhibitor may be MCTA or one or more of the novel compounds described herein; preferably, it is MCTA. It is understood, however, that reference to MCTA in the description that follows applies also to the novel compounds of this invention. While MCTA might perform effectively with any nitrosourea; e.g., chlorozoticin, fotemustine, lomustine, nimustine and ranimustine, at present BCNU, also known as carmustine or, formally, N,N'-bis(2-chloroethyl)-N-nitrosourea, is considered the most active of the group; thus, this invention is directed in particular toward the combination of MCTA with BCNU.

"Comprising" as used herein in connection with "administering" is intended to mean that drugs being administered pursuant to the present invention may be administered as simply a combination of MCTA and BCNU only or may be expanded to include additional drugs which are known or expected to offer additional beneficial characteristics to the combination.

As used herein, "combination" refers to two or more compounds or drugs which are administered simultaneously, sequentially, continuously, intermittently, etc. in accordance with a regimen calculated to take maximum advantage of the characteristics of each of the component drugs. In a preferred embodiment of this invention, the combination comprises MCTA and BCNU. However, combinations of BCNU with two, three and more additional compounds are well known to those skilled in the art and it would be within the spirit and scope of this invention to include one or more of these additional compounds together with the BCNU and MCTA.

"Tumorigenic" activity, as it relates to a cell, refers to both intracellular and extracellular biochemical activity which contributes to the formation of a neoplasm.

A "neoplasm" is an abnormal tissue that grows by cellular proliferation more rapidly than normal and continues to grow even after the stimuli that initiated the new growth cease. A

neoplasm partially or completely lacks structural organization and functional coordination with the normal tissue and usually forms a distinct mass of tissue. Such masses may be benign (benign tumors) or malignant (solid tumor cancer). Malignant neoplasms are locally invasive and destructive and in many cases metastasize (spread to and invade and destroy tissues in areas of the affected organism remote from the site of origin). The process of neoplasm formation is generally referred to as "neoplasia"; i.e., neoplasia is the biochemical process by which a neoplasm forms and grows.

In another aspect of this invention, MCTA and BCNU are used together to inhibit the formation of malignant neoplasms; i.e., to inhibit neoplasia.

With regard to malignant neoplasms, "inhibit" or "inhibiting" refers to eliminating, reducing, containing, impeding, preventing, slowing, retarding and/or restricting neoplasia.

"Statistically significant" means that data obtained from experiments is analyzed using the Student's T Test and "P", the calculated probability that the results obtained are random and not indicative of a true trend, is less than or equal to 0.05 or 5%.

The terms "malignant neoplasm", "cancer", "tumor" and "solid tumor cancer" are used interchangeably herein to refer to the condition well known to those skilled in the art as the life-threatening disease commonly referred to simply as "cancer." Among the most virulent and difficult to treat of the malignant neoplasms are brain tumors. Brain tumors are classified as primary, originating in cells found in the brain itself; secondary, metastatic from sites outside the central nervous system; or developmental, arising from displaced midline epithelium or germ cells. Such tumors are further described as intra-axial or extra-axial. Intra-axial brain tumors are those which originate within the brain parenchyma; that is, the interstitial tissues of the brain itself or

closely associated tissues such as the pineal gland, the posterior pituitary gland, the spinal cord and the retina. Extra-axial tumors, on the other hand, originate in tissues not directly associated with the brain but still within its vicinity; e.g., in the skull, the cranial nerves or in brain appendages such as the pituitary gland. About half of primary intra-axial brain tumors are gliomas which include astrocytoma, particularly anaplastic astrocytoma, glioblastoma multiforme and oligodendroglioma. Both glioblastoma and astrocytoma are expected to be particularly vulnerable to the combination of drugs claimed herein.

About half of the gliomas are glioblastoma multiforme, which will be referred to herein simply as "glioblastoma" and which are generally regarded as the most virulent of all the brain tumors. Glioblastoma consist chiefly of undifferentiated anaplastic cells of glial origin that show marked nuclear pleomorphism, necrosis and vascular endothelial proliferation. They grow extremely rapidly and invade extensively. They occur most frequently in the cerebrum of adults. While survival depends on numerous factors, generally speaking, most patients survive less than 9 months after diagnosis of glioblastoma.

In yet another aspect of this invention, a method is claimed for treating solid tumors (malignant neoplasms) in a patient by administering to the patient a therapeutically effective amount of MCTA and a therapeutically effective amount of a chemotherapeutic nitrosourea, in particular, BCNU. Individual pharmacological compositions of both MCTA and of BCNU, which compositions facilitate the administration of each of the drugs, are an additional feature of this invention. The use of the pharmacological compositions of MCTA and BCNU for the treatment of brain tumors, especially glioblastoma and astrocytoma, is a particularly preferred aspect of this invention.

A "patient" refers to any higher organism which is susceptible to the formation of neoplasms. In particular,

"patient" refers to mammals, especially, of course, human beings.

A "chemotherapeutic" refers to a chemical substance or drug used to treat a disease; the term is most often applied to such substances or drugs which are used primarily for the treatment of cancer.

(1) Biochemistry/Pharmacology of BCNU and MCTA

At present, many chemotherapy agents act by affecting DNA synthesis or function. For example, the alkylating agents, such as cyclophosphamide, directly attack DNA, damaging the molecule so that it cannot perform its cellular function resulting in the death of the cell. The antimetabolites, such as 6-mercaptopurine and 5-fluorouracil interfere with DNA synthesis. Certain antitumor antibiotics such as doxorubicin and bleomycin bind to DNA and thereby prevent the synthesis of RNA. The nitrosoureas, of which BCNU is currently considered the most potent, act similarly to the alkylating agents. BCNU has been postulated to participate in cross-linking both DNA and DNA-protein. In addition, the nitrosoureas appear to inhibit changes necessary for repair of damaged DNA, perhaps by virtue of the fact that, as well as being alkylating agents, the nitrosoureas are carbamoylating agents since isocyanates are formed during their decomposition at physiological pHs; proteins are known to be carbamoylated by isocyanates derived from BCNU.

BCNU has been used to treat a number of different solid tumor cancers; e.g., lymphomas, malignant melanoma, breast cancer and multiple myeloma. It has been employed in combination with other drugs such as cyclophosphamide, cisplatin, vincristine, melphalan, dexamethasone, vinblastine, methotrexate, daunorubicin, etoposide, procarbazine, prednisone, cytarabine, thioguanine and hydroxyurea. In addition, because of its ability to cross the blood-brain barrier, BCNU is presently the chemotherapeutic of choice for

the treatment of brain cancers, including glioblastoma. Although combinations of BCNU with other chemotherapeutic agents are used in the treatment of gliomas as well as other neoplasms, the number of such combinations is fewer than for non-brain tumors due to the inability of many other chemotherapeutic agents to cross the blood-brain barrier. For example, for the treatment of gliomas, BCNU has been combined with cyclophosphamide, cisplatin, thiotepa, procarbazine, vincristine, etoposide and taxol.

A limiting factor for the use of BCNU, alone or in combination with other drugs, is the inherent toxicity of the known chemotherapeutic agents. BCNU is known to cause myelosuppresion (resulting in leukopenia and thrombocytopenia), pulmonary fibrosis (lung disease), hepatotoxicity (liver damage) and nephrotoxicity (kidney damage). The effects of BCNU are dose dependent, progressive and cumulative. In addition, BCNU itself has been shown to cause tumors in rats and mice at doses approximating those clinically employed (Bristol Laboratories Oncology Products, FDA disclosure enclosure accompanying commercial BCNU packaging).

Reducing the incidence of drug-related adverse reactions from BCNU by reducing the dose through combination therapy with existing chemotherapeutic agents is only marginally effective. At stated previously, the most commonly used drugs in combination with BCNU for the treatment of gliomas are other alklyating agents; for the most part they exhibit toxicities similar to that of BCNU. For example, a complication associated with cisplatin is the possibility of renal (kidney) failure. Cyclophosphamide has been associated with mucositis (van der Wall, et al., Cancer Treatment Reviews, 21(2):105-142 (1995)). In one study, treatment with thiotepa and etoposide in combination with BCNU (total dosage BCNU: 600 mg/m^2 over three days) has been reported to result in high mortality due to multiorgan failure (Finlay, Bone Marrow Transplantation, 18 (Suppl. 3):S1 - S5 (1996)).

Thus, what is needed is a compound (or compounds) whose anti-tumor activity coordinates with that of BCNU but which does not concomitantly increase overall toxicity to the patient. We have found that MCTA is such a compound.

MCTA has been shown to be well-tolerated in human studies. For instance, there were few side effects among 500 rheumatoid arthritis patients treated with MCTA in a Phase II clinical study (Bartlett, et al., Non-steroidal Anti-inflammatory Drugs: Mechanisms and Clinical Uses, Lewis, AJ, Furst, DE, eds., New York:Dekker ,1994:349). In two studies of the effect of MCTA against a variety of solid tumors including ovarian, prostate and non-small cell lung cancer, MCTA was reported to be only mildly to moderately toxic at the highest doses employed (Rosen, et al., Proc. Annu. Meet. Am. Soc. Clin. Oncol., 16:A739 (1997); VanUmmersen, et al., Proc. Annu. Meet. Am. Soc. Clin. Oncol., 16:A740 (1997)). Furthermore, in a clinical trial in which the effect of MCTA against malignant gliomas, which cell lines were found to be the most sensitive to the compound in pre-clinical trials, was studied, no dose-limiting toxicities were observed (Malkin, et al., Proc. Annu. Meet. Am. Soc. Clin. Oncol., 16:A1471 (1997)).

MCTA, however, affects tumors by a mode of action which to the inventors' knowledge is completely different from that of the known chemotherapeutic agents and, therefore, it was unknown whether it would have any beneficial effect in conjunction with BCNU. That is, as stated previously, BCNU acts primarily as an alkylating agent, attacking and damaging a number of key enzymatic reactions involved in DNA synthesis and DNA itself. Virtually all of the reported chemotherapeutic combinations with BCNU likewise interfere directly in one way or another with DNA synthesis or activity. MCTA, on the other hand, does not interact directly with DNA synthesis/activity. Rather, MCTA intervenes at an earlier stage of the cell cycle. That is, MCTA has been shown to inhibit signaling events which are necessary for a cell to initially enter into the active

stage of DNA synthesis and cell proliferation where BCNU and the other alkylating agents exhibit their predominant activity. Specifically, MCTA inhibits platelet derived growth factor (PDGF) mediated cell signaling events which are necessary for the inception of cell growth, cell cycle progression and cell proliferation. When treated with MCTA, cells become essentially quiescent, at least with regard to DNA synthesis and cell division (mitosis), and remain so until treatment with MCTA is withdrawn at which time cell activity resumes. The fact that a cell will return to normal activity once treatment with MCTA is terminated demonstrates that MCTA, unlike BCNU and other alkylating agents, is cytostatic rather than cytotoxic. That is, MCTA inhibits cell activity but does not kill cells.

Many types of tumor cells have been shown to over-express PDGF receptors and to be particularly dependent on the abnormally high PDGF signaling events arising from this over-expression for their continued proliferation. For example, PDGF ligands and receptors have been detected in many cell lines or tissues derived from, without limitation, glioma, melanoma, esophageal tumors, gastric tumors, colorectal adenocarcinoma, basal cell cancer, choriocarcinoma, breast cancer, Kaposi's sarcoma, ovarian cancer, non-small cell lung carcinoma and prostate adenocarcinoma. Some cancer cells have even been shown to co-express PDGF itself as well as the receptor for it. This suggests that these tumor cells may be self-stimulating; i.e., employ an autocrine as well as a paracrine growth mechanism which could account for the previously noted propensity for neoplasms to continue growing and proliferating even when the external stimuli are removed. MCTA, however, is a blocker of PDGF-mediated activity and thereby is capable of inhibiting the uncontrolled growth and proliferation of tumors.

Given the lack of any information regarding a combination of BCNU, a DNA synthesis inhibitor, with a compound such as MCTA which effectively prevents a cell from entering the stage

of cell cycle where DNA synthesis occurs, the excellent results obtained from the combination of the two compounds was truly surprising. MCTA alone was efficacious at concentrations of 20, 10 and 5 mg/kg/day. BCNU alone was effective at 27 and 18 mg/kg. BCNU alone at 12 mg/kg was ineffective. However, the combination of 5 mg/kg of MCTA and 12 mg/kg BCNU showed a statistically significant percentage inhibition as high as 63.4% on day 18. In addition, 5 mg MCTA with 18 mg/kg BCNU also exhibited a greater percentage inhibition than the corresponding amount of MCTA or BCNU alone, as did 10 mg/kg of MCTA with both 12 and 18 mg/kg BCNU. Furthermore, the increased effect was of longer duration than that afforded by BCNU alone as indicated by the increased number of days of statistically significant percentage inhibition.

Finally, there was no observed change in the mortality of the mice when the combination of BCNU and MCTA was used, indicating that there was no additive general toxicity even though there was a distinct therapeutic advantage from the concentration. This suggests that the therapeutic index, that is, the ratio of the LD_{50} (the dose lethal to 50% of the population) to the ED_{50} (the dose therapeutically effective in 50% of the population), of the MCTA/BCNU combination is higher that for either drug alone which translates into a substantial benefit to patients in need of as much chemotherapy as possible without a concomitant increase in intolerable side effects requiring cessation of treatment.

It should be noted that MCTA is known to metabolize in mammals to N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxy-crotonamide. Generally speaking, MCTA tends to metabolize most readily when subjected to acid conditions such as those encountered in the stomach when a drug is administered orally. However, the preferred mode of administration of the combination claimed herein is more direct; e.g., without limitation, intravenously or intraarterially. Nevertheless, a portion of the MCTA administered to a patient may metabolize

to N-(4-trifluoro- methylphenyl)-2-cyano-3-hydroxy- crotonamide prior to reaching the target tumor. Therefore, some of the activity of the claimed combination may in fact be due to a combination which includes not only MCTA and BCNU but N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotonamide as well. The mode of action of N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotonamide has been determined to be inhibition of pyrimidine biosynthesis and therefore subsequent cell growth and/or survival which may in fact provide additional synergy of the claimed combination. Thus, it is understood that the scope and spirit of this invention encompasses not only MCTA and BCNU in the claimed combination but N-(4trifluoromethylphenyl)-2-cyano-3-hydroxycrotonamide, the metabolite of MCTA, as well.

G. Pharmacological Compositions and Therapeutic Applications.

(1) General

MCTA as well as the compounds disclosed herein are preferably administered to a patient in a pharmaceutical composition comprising one or more compounds of this invention together with pharmaceutically acceptable carrier(s) and/or excipients. The compound can be prepared as a physiologically acceptable salt (*i.e.*, non-toxic salts which do not prevent the compound from exerting its effect).

Physiologically acceptable salts can be acid addition salts such as those containing hydrochloride, sulfate, phosphate, sulfamate, acetate, citrate, lactate, tartrate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, cyclohexylsulfamate and quinate. Such salts can be derived using acids such as hydrochloric acid, sulfuric acid, phosphoric acid and sulfamic acid, acetic acid, citric acid, lactic acid, tartaric acid, malonic acid, methanesulfonic acid, ethanesulfonic acid, benzenesulfonic

acid, p-toluenesulfonic acid, cyclohexylsulfamic acid, and quinic acid.

Physiologically acceptable salts can be prepared by standard techniques. For example, the free base form of the compound is first dissolved in a suitable solvent such as an aqueous or aqueous-alcohol solution, containing the appropriate acid. The salt is then isolated by evaporating the solution. In a another example, the salt is prepared by reacting the free base and acid in an organic solvent.

Carriers or excipient can be used to facilitate administration of the compound, for example, to increase the solubility of the compound. Examples of carriers and excipient include calcium carbonate, calcium phosphate, various sugars or starches, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols and physiologically compatible solvents. The compositions or pharmaceutical composition can be administered by different routes including intravenously, intraperitoneal, subcutaneous, and intramuscular, orally, topically, or transmuccosally.

The specific delivery route of any selected agent depends on the use of the agent. Generally, a specific delivery program for each agent focuses on agent uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies include uptake assays to evaluate, *e.g.*, cellular nucleic acid or protein uptake, regardless of the delivery vehicle or strategy. Such assays also determine the intracellular localization of the agent following uptake, ultimately establishing the requirements for maintenance of steady-state concentrations within the cellular compartment containing the target sequence (nucleus and/or cytoplasm). Efficacy and cytotoxicity can then be tested. Toxicity not only includes cell viability but also cell function. Generally, the dosages

of the mutated protein and nucleic acid is as described above for the featured compounds.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles falling into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Pumps can also be used for this purpose.

From the above group of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion to those lipids making up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Antibodies can be attached to liposomes to target particular cells.

Topical administration of the featured compound is advantageous, particularly when treating skin disorders such as Kaposi's sarcoma, since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the agent to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material applied is far less than that required for other administration routes.

Many compounds, MCTA for example, are preferably absorbed systemically when used to treat disorders such as cancer. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption

include: intravenous, subcutaneous, intraperitoneal, intranasal and intrathecal. Each of these administration routes expose the drug to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size.

MCTA is hydrophobic and thus not very soluble in water. It is expected that the novel compounds of this invention may also be hydrophobic. Effective doses of hydrophobic compounds for systemic administration can be obtained using the pharmaceutical formulations described in US Patent No. 5,610,173, issued March 11, 1997. A particularly preferred formulation is obtained using a combination of the compound and VPD:D5W. VPD consists of a solution of 12% w/v polysorbate 80, 0.55% citric acid (anhydrous), 35% w/v polyenthlene glycol (MW = 300 daltons) and 26.3% v/v 190 proof ethanol. VPD is diluted 1:22 in a diluent. Preferred diluents are 0.45% saline, and 0.9% saline. A particularly preferred diluent is 5% dextrose in water (D5W).

Another way of overcoming the hydrophobicity problem includes the use of frequent small daily doses rather than a few large daily doses. For example, the composition can be administered at short time intervals, preferably the composition can be administered using a pump to control the time interval or achieve continuously administration. Suitable pumps are commercially available (e.g., the ALZET® pump sold by Alza corporation, and the BARD ambulatory PCA pump sold by Bard MedSystems).

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars,

including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

The proper dosage depends on various factors such as the type of disease being treated, the particular composition being used, the dosing regimen and the size and physiological condition of the patient. For the treatment of cancers it is prefered that the minimal plasma concentration in a patient be greater than 5 µg/ml, more preferably greater than 25 µg/ml, most preferably greater than 50 µg/ml. The compound can be delivered daily or less frequently provided plasma levels of

the active moiety are sufficient to maintain therapeutic effectiveness. Plasma levels may be reduced if pharmacological effective concentrations of the drug are achieved at the site of interest.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. For example, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} as determined in cell culture (i.e., the concentration of the test compound which achieves a half-maximal disruption of the protein complex, or a half-maximal inhibition of the cellular level and/or activity of a complex component). Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by HPLC.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p1).

It should be noted that the attending physician would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administrated dose in the management of the oncogenic disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above may be used in veterinary medicine.

(2) MCTA/BCNU

MCTA and BCNU can be administered to a human patient per se, or in pharmacological compositions wherein they are mixed with suitable carriers or excipient(s). In general, techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

(i) Routes Of Administration.

Suitable routes of administration may, for example, include transmucosal, intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraarterial or intraperitoneal.

Alternately, one may administer MCTA and BCNU in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

Other means of administering the drugs of this invention which are well within the knowledge of those skilled in the art are within the scope and spirit of this invention.

(ii) Composition/Formulation.

Pharmacological compositions of MCTA and BCNU are preferred embodiments of this invention. Pharmacological compositions of the present invention may be manufactured by processes well known in the art; e.g., by means of conventional mixing, dissolving, granulating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmacological compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, preferred formulation for BCNU are provided with the commercial compound. In one instance this includes Dehydrated Alcohol injection USP in which the BCNU is dissolved and then the alcoholic solution is diluted aseptically with sterile water to a final concentration of 3.3 mg/ml of BCNU in 10% ethanol, pH 5.6 to 6.0 (BCiNU® from Bristol Laboratories Oncology Products).

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The compounds may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous

vehicles, and may contain formulating agents such as suspending, stabilizing and/or dispersing agents.

For MCTA, a preferred formulation for injection comprises ethanol/water containing a surfactant. The specifics of such formulation are disclosed in Schwartz, et al., U. S. Patent No. 5,610,173, issued March 11, 1997, which patent is incorporated by reference as if fully set forth herein.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmacological compositions herein also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

(iii) Dosage.

Pharmacological compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of compound effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein and, with regard to BCNU, existing literature.

For MCTA, the dosage and interval should be adjusted individually to provide plasma levels of the compound at or above the amount necessary to maintain the PDGF-mediated signal transduction inhibiting effects of the compound. HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals for MCTA can also be determined using plasma concentrations. MCTA should be administered using a regimen which maintains plasma levels above, preferably well above, the level necessary to elicit its therapeutic effect for 10-90% of the time, preferably between 50-90% and most preferably between 70-90%.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of both MCTA and BCNU administered will, of course, be dependent on the subject being treated, on the subject's weight (from which body surface area is calculated), the severity of the cancer, the manner of administration and the judgment of the prescribing physician.

Since MCTA is cytostatic rather than cytotoxic, it is a feature of this invention that MCTA be administered to a patient throughout all or a substantial portion of a course of treatment with the combination of MCTA and BCNU. A "course of treatment" is defined as a time period over which a complete cycle of a pre-determined regimen of administration of MCTA and BCNU is performed.

With regard to the pre-determined regime of a course of treatment, such regime may entail simultaneous administration of MCTA and BCNU or sequential administration of the drugs in such order, dosages and intervals as are deemed most appropriate with regard to a particular patient by the treating physician. Or, MCTA may be administered prior to administration of BCNU. Conversely, BCNU can be administered before treatment with MCTA begins. Preferable, however, MCTA

is administered to the patient in a "loading dose" prior to administration of BCNU.

As used herein, a "loading dose" refers to an initial dose or doses of a drug given to create an initial level of the drug in the body of the patient prior to the administration of another drug. The loading dose may be administered as a single dose, as a continuous infusion over time or as several discrete administrations of equal or unequal doses at discrete intervals. The time period over which the loading dose is administered may vary as seen fit by the prescribing physician. Preferable, however, the loading dose is administered over a period of 3 to 6 days either as a continuous infusion or as equal doses given on each day of the loading dose period. The loading dose may be from 100 to 550 $mg/m^2/day$. Preferable, the loading dose is 200 to 500 $mg/m^2/day$. Most preferably, it is from 350 - 450 $mg/m^2/day$. The exact amount of the loading dose would, of course, be determined by the treating physician on a case-by-case basis.

In a preferred embodiment of this invention BCNU is administered on the day immediately following completion of the loading dose. The BCNU may be administered in a single dose or in smaller doses given over a period of several days. The amount of BCNU administered may be from 75 to 200 mg/m^2. Actual choice of dosage is likewise within the province of the treating physician. Preferable, however, the BCNU is administered in a single dose of from 150 to 200 mg/m^2.

The overall course of treatment may vary extensively depending on the judgment of the treating physician. Preferable it is from 6 to 8 weeks. During the course of treatment, maintenance doses of MCTA are administered either for the entire duration of the course of treatment or for any portion thereof. Such maintenance doses may be administered at intervals of from 2 to 10 days apart. Preferable, the maintenance doses are administered 6-7 days apart and the number of such maintenance doses is from 5 to 10, preferable

at a rate of one maintenance dose per week for the entire course of treatment; e.g., if an 8 week course of treatment is selected, a maintenance dose of MCTA is administered during each of those weeks at approximately one-week intervals. The amount of the maintenance dose would be selected by the treating physician but is preferably in the range of 100 to 555 mg/m^2/week, more preferable 200 to 450 mg/m^2/week and most preferably, from 350 to 450 mg/m^2/week.

A "maintenance dose" refers to an additional dose or doses of the drug given initially as a loading dose, the purpose being to maintain the level of the drug or its metabolite achieved by the loading dose for an extended period of time.

At the conclusion of a course of treatment, the patient is evaluated and, if the indications, including but not limited to, positive response to the therapy and no signs of serious side effects, are appropriate in the eyes of the treating physician, the course of treatment may be repeated. The number of repeat courses of treatment is left to the discretion of the treating physician and should be limited only by the determination by the treating physician that continued treatment could be detrimental to the patient.

It is of course to be understood that dosages different from those described above may be administered and still be within the scope and spirit of this invention. For instance, different routes of administration, such as direct injection into a tumor or interstitial implantation would be expected to permit even lower doses than those discussed herein due to less likelihood of dilution and metabolic decomposition when the target is more directly addressed. It would be most beneficial to be able to lower the dosage of the more individually toxic of the two drugs of the combination, BCNU, with 50 - 100 mg/m^2 below the dosage usually recommended by the commercial suppliers of the drug as a reasonable target. In addition, as discussed previously herein, employing other known

chemotherapeutic agents along with the core duo of MCTA and BCNU could permit lower dosages of BCNU concomitant with equally low doses of the added drug. Again, so long as the unique combination of MCTA with a nitrosourea is part of the combination therapy, the combination is within the scope and spirit of this invention.

The unexpected synergistic activity of the combination of MCTA with BCNU without apparent increase in overall toxicity suggests that MCTA might also work in combination with other chemotherapeutic agents as well. For instance, the combination of MCTA with other alkylating agents might likewise afford synergistic activity without concomitant increased toxicity. Such alkylating agents could include, without limitation, the alkyl sulfonates; e.g., busulfan (used for treatment of chronic granulocytic leukemia), improsulfan and piposulfan; the aziridines; e.g., benzodepa, carboquone, meturedepa, and uredepa; the ethyleneimines and methylmelamines; e.g., altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphoramide and trimethylolmelamine and the nitrogen mustards; e.g., chlorambucil (used in treatment of chronic lymphocytic leukemia, primary macroglobulinemia and non-Hodgkin's lymphoma), cyclophosphamide (used in treatment of Hodgkin's disease, multiple myeloma, neuroblastoma, breast cancer, ovarian cancer, lung cancer, Wilm's tumor and rhabdomyosarcoma), estramustine, ifosfamide, novembrichin, prednimustine and uracil mustard (for primary thrombocytosis, non-Hodgkin's lymphoma, Hodgkin's disease and ovarian cancer); and the triazines; e.g., dacarbazine (used for soft-tissue sarcoma).

Likewise, MCTA could have a beneficial effect in combination with the antimetabolite chemotherapeutic agents such as, without limitation, the folic acid analogs (e.g., methotrexate (used in treating acute lymphocytic leukemia, choriocarcinoma, mycosis fungoides, breast, neck and head and lung cancer, osteogenic sarcoma) and pteropterin); the

pyrimidine analogs (fluorouracil (5-FU) used in treatment of breast, colon, stomach, pancreatic, ovarian, head and neck, and urinary cancers as well as topically for premalignant skin lesions and cytarabine (acute granulocytic and acute lymphocytic leukemias) and the purine analogs such at mercaptopurine and thioguanine which find use in the treatment of acute granulocytic, acute lymphocytic and chronic granulocytic leukemias).

MCTA could also prove effective in combination with natural product chemotherapeutic agents such as, without limitation, the vinca alkaloids (vinblastine (used for breast and testicular cancer), vincristine and vindesine), the epipodophylotoxins (etoposide, teniposide (both used in the treatment of testicular cancer and Kaposi's sarcoma)), the antibiotic chemotherapeutic agents (daunorubicin, doxorubicin, bleomycin mitomycin (used for stomach, cervix, colon, breast, bladder and pancreatic cancer)), dactinomycin, plicamycin, bleomycin (used for skin, esophagus and genitourinary tract cancer) and the enzymatic chemotherapeutic agents such as L-Asparaginase.

Based on the disclosures of this invention, MCTA might also benefit the activity of chemotherapeutic agents such as platinum coordination complexes (cisplatin, etc.), substituted ureas (hyroxyurea), methylhydrazine derivatives (procarbazine), adrenocortical suppressants (mitotane, aminoglutethimide) as well as hormones and antagonists such as adrenocorticosteroids (prednisone), progestins (hydroxyprogesterone caproate), estrogens (diethylstilbestrol), antiestrogens (tamoxifen) and androgens (testosterone propionate).

Finally, the combination of MCTA with mitoxantrone or paclitaxel might be expected to display especially beneficial results in the treatment of solid tumors.

H. Measurement Of Cell Toxicity

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index: IC_{50}/LD_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques (Mossman, 1983, J. Immunol. Methods, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, J. Immunol. Methods, 64:314; Decker and Lohmann-Matthes, 1988, J. Immunol. Methods, 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

Synthesis

The compounds of this invention may be readily synthesized using techniques well known in the chemical arts. The following syntheses are shown by way of example only and are not to be construed as limiting in any way. In fact, it will be appreciated by those skilled in the art that other synthetic pathways for forming the compounds of the invention are available and that the following are but a few of the possible routes to the claimed compounds.

a. 3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] isoxazole

Ethyl propiolate (2.8 g) and pyrrolidine (1.4 g) in 5 ml of acetonitrile are mixed at room temperature for 1 hour, the solvent evaporated and the ethyl 3-pyrrolidin-1-acrylate used as isolated or distilled under vacuum. Triethylamine (0.25 ml) is added to a mixture of 1.8 g of ethyl 3-pyrrolidin-1-acrylate, 0.9 g of nitroethane and 2.5 g of phenyl

isothiocyanate in 10 ml of toluene at room temperature and stirred overnight. The mixture is then refluxed for 0.5 hour, cooled, and the diphenylurea removed by filtration. The mixture is washed with water and brine, dried over anhydrous sodium sulfate, and evaporated to dryness under vacuum to give 1.4 g of ethyl 3-methyl-4-isoxazolecarboxylate. (Stork, G., McMurry, J. C., J. Am. Chem. Soc. 89, 5461,1967).

Ethyl 3-methyl-4-isoxazolecarboxylate (1.3 g) is stirred at room temperature overnight in 5 ml of ethanol and 10 ml of 2.5 N sodium hydroxide. Dilution with water, cooling in ice, and acidification to pH 2 with 6 N hydrochloric acid precipitates an off-white solid which is collected by vacuum filtration, washed with ethanol/water, and dried under vacuum to give 1.0 g of 3-methyl-4-isoxazolecarboxylic acid.

3-Methyl-4-isoxazolecarboxylic acid (0.9 g) is stirred with 5 ml of thionyl chloride at room temperature for one hour and the mixture evaporated to dryness. The residue is dissolved in 5 ml tetrahydrofuran and 1 ml of pyridine containing 1.2 g of 4-trifluoromethylaniline and stirred overnight. The mixture is refluxed for one hour, cooled and diluted with water to give an off-white precipitate. The solid is collected by vacuum filtation, washed with ethanol/water and dried under vacuum to give 1.2 g of 3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole.

b. 3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] isoxazole

A solution of 5.5 g of 4-(trifluoromethyl)aniline in 6 ml of toluene at 120 °C is treated with 5.4 g of 2,2,6-trimethyl-4H-1,3-dioxin-4-one. The mixture is refluxed for four hours and cooled. The precipitate is collected by vacuum filtration, washed with toluene and dried to give 6 g of N-[4-(trifluoromethyl)phenylacetoacetamide.

N-[4-(Trifluoromethyl)phenylacetoacetamide (6 g), 4 g of triethylorthoformate and 8 g of acetic anhydride is cautiously

heated to reflux for 2 hours. The reaction is cooled to room temperature and the precipitate collected by vacuum filtration and dried to give 4 g of N-[4-(trifluoromethyl)phenyl]-2-(ethoxymethylene)acetoacetamide.

N-[4-(Trifluoromethyl)phenyl]-2-(ethoxymethylene)-acetoacetamide (4 g) is suspended in 10 ml of ethanol treated with 1.5 g of hydroxylamine hydrochloride in 10 ml of water which has been adjusted to pH 5 with sodium hydroxide. The mixture is stirred and warmed to 40 °C for 2 hours, then cooled to room temperature and the precipitate collected by vacuum filtration. Sodium hydroxide (1 g) is added to the filtrate which is stirred for 30 minutes. The mixture is acidified to pH 2 with 6 N hydrochloric acid and the precipitate collected by vacuum filtration. The filtrate is then diluted with 100 ml of water and allowed to stand at 4 °C overnight. The precipitate is collected by vacuum filtration, washed with ethanol:water 2:1 and dried to give 300 mg of crude 3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole. The crude is purified on a column of silica gel eluting with ethyl acetate:hexane 1:4 to give 100 mg of 3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, an off-white solid.

c. 3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] pyrazole

N-[4-(Trifluoromethyl)phenyl]-2-(ethoxymethylene) acetoacetamide (1 g) in 3 ml of ethanol is stirred overnight with 0.3 g of hydrazine hydrate. The precipitate is collected by vacuum filtration, washed with ethanol:water 2:1 and dried to give 0.3 g of 3-methyl-4-[4-(trifluoromethyl) phenylaminocarbonyl]pyrazole as an off-white solid.

d. 3-Methyl-4-(pyrid-2-aminocarbonyl)isoxazole

By substituting 2-aminopyridine for 4-(trifluoromethyl) aniline in a, the identical process gives 1 g of 3-methyl-4-(pyrid-2-aminocarbonyl)isoxazole as an off-white solid.

e. 3-Methyl-4-(pyrid-2-aminocarbonyl)pyrazole

By substituting 2-aminopyridine for 4-(trifluoromethyl) aniline in the first two steps of the process of b, the identical process gives 2.5 g of N-(pyrid-2-yl)-2-(ethoxymethylene)acetoacetamide. By substituting N-(pyrid-2-yl)-2-(ethoxymethylene)acetoacetamide for N-[4-(trifluoromethyl)phenyl]-2-ethoxymethylene)acetoacetamide in c, the identical process gives 0.3 g of 3-methyl-4-(pyrid-2-aminocarbonyl)pyrazole as an off-white solid.

f. 3-Cyclopropyl-4-[4-(trifluoromethyl)phenylamino-carbonyl]isoxazole

By substituting cyclopropylnitromethane (Williams et al (1965) J. Org. Chem. 30: 2674-2675) for nitroethane in a, the identical process gives 1.2 g of 3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole as an off white solid.

g. 3-Cyclopropyl-4-[4-(trifluoromethyl)phenylamino-carbonyl]pyrazole

Methyl cyclopropylcarbonylacetate (1.4 g) and 1.6 g of 4-(trifluoromethyl)aniline are heated to 160 °C overnight. When the crude product (or the product purified by crystallization or silica gel chromatography) is substituted for N-[4-trifluoromethylphenyl]acetoacetamide in the second step of b, the identical process gives N-[4-trifluoromethylphenyl]-2-(ethoxymethylene)-cyclopropylcarbonylacetamide. When N-[4-trifluoromethylphenyl]-2-(ethoxymethylene)cyclopropylcarbonyl-acetamide is substituted for N-[4-(trifluoromethyl)phenyl]-2-(ethoxymethylene)-acetoacetamide in c, the identical process

gives 3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] pyrazole.

h. 3-Cyclopropyl-4-(pyrid-2-aminocarbonyl)isoxazole

When 2-aminopyridine is substituted for 4-(trifluoromethyl)aniline in f, the identical process gives 3-cyclopropyl-4-(pyrid-2-aminocarbonyl)isoxazole.

i. 3-Cyclopropyl-4-(pyrid-2-aminocarbonyl)pyrazole

When 2-aminopyridine is substituted for 4-(trifluoromethyl)aniline in g, the identical process gives 3-cyclopropyl-4-(pyrid-2-aminocarbonyl)pyrazole.

j. 3-Methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole

3-Methyl-4-[4-(trifluoromethylsulfonyl)phenylamino carbonyl] isoxazole

By substituting 3-methyl-4(trifluoromethyl)aniline (Tordeaux, M., et al (1990) J. Chem. Soc. Perkin Trans. EN 8: 2293-2299) and 4-(trifluoromethylsulfonyl)aniline (Jagupolski, M. (1954) Zh. Obshch. Khim. 24: 887-893, Engl. Ausg. S. 885-889)for 4-(trifluoromethyl)aniline in the identical process of a, 3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole and 4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole are prepared.

k. 3-Methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole

3-Methyl-4-[4-(trifluoromethylsulfonyl)phenylamino carbonyl]pyrazole

By substituting the appropriate amine for 4-(trifluoromethyl)aniline in the identical process of g, 3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole and 3-Methyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl] pyrazole are prepared.

l. 3-Allyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] isoxazole;

3-Allyl-4-(pyrid-2-aminocarbonyl)isoxazole;

3-Allyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole;

3-Allyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole.

By substituting 4-nitro-1-butene (Seebach, D., et al (1978) Angew. Chem. GE 90: 479-480) for nitroethane in a, and then the appropriate amines for 4-(trifluoromethyl)aniline in a, the identical process gives 3-allyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, 3-allyl-4-(pyrid-2-aminocarbonyl)isoxazole, 3-allyl-4-[3-methyl-4-(trifluoromethyl) phenylaminocarbonyl]isoxazole and 3-allyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole.

m. 3-Allyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]pyrazole

3-Allyl-4-(pyrid-2-aminocarbonyl)pyrazole;

3-Allyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole;

3-Allyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]pyrazole.

By substituting methyl 3-butenoate for methyl cyclopropylcarbonylacetate in g, and then the appropriate amines for 4-(trifluoromethyl)aniline in g, the identical process gives 3-allyl-4-[4-(trifluoromethyl) phenylaminocarbonyl] pyrazole, 3-allyl-4-(pyrid-2-aminocarbonyl)pyrazole), 3-allyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl] pyrazole, and 3-allyl-4-[4-(trifluoromethylsulfonyl) phenylaminocarbonyl]pyrazole.

n. 3,5-Dimethyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole

3,5-Dimethyl-4-(pyrid-2-aminocarbonyl)isoxazole;

3,5-Dimethyl-4-[3-methyl-4-(trifluoromethyl)phenylamino-carbonyl]isoxazole;

3,5-Dimethyl-4-[4-(trifluoromethylsulfonyl)phenylamino-carbonyl]-isoxazole.

By using 3,5-dimethyl-4-chlorocarbonylisoxazole or by substituting ethyl methylpropiolate for ethyl propiolate in a and then the appropriate amines for 4-(trifluoromethyl)aniline in a, the identical process gives 3,5-dimethyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole , 3,5-dimethyl-4-(pyrid-2-aminocarbonyl)isoxazole, 3,5-dimethyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole (Example 25), and 3,5-dimethyl-4-[4-(trifluoromethylsulfonyl)phenylamino-carbonyl]isoxazole.

o. 3,5-Dimethyl-4-[4-(trifluoromethyl)phenylamino-carbonyl]pyrazole

3,5-Dimethyl-4-(pyrid-2-aminocarbonyl)isoxazole;

3,5-Dimethyl-4-(pyrid-2-aminocarbonyl)pyrazole;

3,5-Dimethyl-4-[3-methyl-4-(trifluoromethyl)phenyl-aminocarbonyl]pyrazole;

3,5-Dimethyl-4-[4-(trifluoromethylsulfonyl)phenylamino-carbonyl]pyrazole.

By substituting triethylorthoacetate for triethylortho-formate in the second step of b, and then substituting the appropriate amines for 4-(trifluoromethyl) aniline in b, the identical process gives the corresponding substituted 2-(ethoxymethylene)acetoacetamides. By substituting the corresponding substituted 2-(ethoxymethylene)acetoacetamides for N-[4-(trifluoromethyl)phenyl]-2-(ethoxymethylene)aceto-acetamide in c, the identical process gives 3,5-dimethyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] pyrazole, 3,5-dimethyl-4-(pyrid-2-aminocarbonyl)pyrazole, 3,5-dimethyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl] pyrazole, and 3,5-dimethyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl] pyrazole.

p. 5-(2-Chlorophenyl)-3-methyl-4-[4-(trifluoromethyl)-phenylaminocarbonyl]isoxazole

5-(2-chlorophenyl)-3-methyl-4-(pyrid-2-aminocarbonyl) isoxazole;

5-(2-chlorophenyl)-3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole;

5-(2-Chlorophenyl)-3-methyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole.

By substituting ethyl 2-chlorophenylpropiolate (Newman, M. (1955) J. Amer. Chem. Soc. 77:5549) for ethyl propiolate in a, and then the appropriate amines for 4-(trifluoromethyl)aniline in a, the identical process gives 5-(2-chlorophenyl)-3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, 5-(2-chlorophenyl)-3-methyl-4-(pyrid-2-aminocarbonyl)isoxazole, 5-(2-chlorophenyl)-3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, 5-(2-chlorophenyl)-3-methyl-4-[4-(trifluoromethylsulfonyl)-phenylaminocarbonyl]isoxazole.

q. 5-(2-Chlorophenyl)-3-methyl-4-[4-(trifluoromethyl)-phenylaminocarbonyl]pyrazole

5-(2-chlorophenyl)-3-methyl-4-(pyrid-2-aminocarbonyl) pyrazole;

5-(2-chlorophenyl)-3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole;

5-(2-Chlorophenyl)-3-methyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]pyrazole.

By substituting methyl 2-chlorobenzoylacetate for methylcyclopropylcarbonylacetate in g, and then substituting the appropriate amines for 4-(trifluoromethyl)aniline in g, the identical process with triethylorthoacetate substituted for triethylorthoformate in b, gives 5-(2-chlorophenyl)-3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]prazole, 5-(2-chlorophenyl)-3-methyl-4-(pyrid-2-aminocarbonyl)pyrazole, 5-(2-chlorophenyl)-3-methyl-4-[3-methyl-4-(trifluoromethyl)-

phenylaminocarbonyl]pyrazole and 5-(2-chlorophenyl)-3-methyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]pyrazole.

r. 5-(2-Chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole

5-(2-Chlorophenyl)-3-cyclopropyl-4-(pyrid-2-aminocarbonyl)isoxazole;

5-(2-Chlorophenyl)-3-cyclopropyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole;

5-(2-chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole.

By substituting ethyl 2-chlorophenylpropiolate for ethyl propiolate and cyclopropylnitromethane for nitroethane in a, and then the appropriate amines for 4-(trifluoromethyl)aniline in a, the identical process gives 5-(2-chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] isoxazole, 5-(2-chlorophenyl)-3-cyclopropyl-4-(pyrid-2-aminocarbonyl)isoxazole, and 5-(2-chlorophenyl)-3-cyclopropyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole and 5-(2-chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]isoxazole.

s. 5-(2-Chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]pyrazole

5-(2-chlorophenyl)-3-cyclopropyl-4-(pyrid-2-aminocarbonyl)pyrazole;

5-(2-Chlorophenyl)-3-cyclopropyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole;

5-(2-chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]pyrazole.

By substituting the appropriate amines for 4-(trifluoromethyl)aniline in g, and then by substituting triethyl-2-chloroorthobenzoate for triethylorthoformate in b, the identical process of g gives 5-(2-chlorophenyl)-3-cyclopropyl-4-[4-(trifluoromethyl)phenylaminocarbonyl] pyra-

zole, 5-(2-chlorophenyl)-3-cyclopropyl-4-(pyrid-2-amino-carbonyl) pyrazole, and 5-(2-chlorophenyl)-3-cyclopropyl-4-[3-methyl-4-(trifluoromethyl) phenylaminocarbonyl]pyrazole and 5-(2-chlorophenyl)-3-cyclopropyl-4-[3-methyl-4-(trifluoromethyl-sulfonyl)phenylaminocarbonyl]pyrazole.

t. 3-(2-Carboxyethyl)-4-[4-(trifluoromethyl)phenylamino-carbonyl]isoxazole

3-(2-carboxy- ethyl)-4-(pyrid-2-aminocarbonyl)isoxazole;

3-(2-Carboxyethyl)-4-[3-methyl-4-(trifluoromethyl)-phenylaminocarbonyl]isoxazole;

3-(2-Carboxyethyl)-[4-(trifluoromethylsulfonyl) phenylaminocarbonyl]isoxazole.

By substituting ethyl 4-(t-butoxycarbonylethyl) nitrobutane (from 4-nitrobutyric acid methyl ester, Bissell, E. R. et al., Tetrahedron (1970), p 5737 - 5743) for ethyl propiolate in a, and then the appropriate amines for 4-(trifluoromethyl)aniline in a, the identical process followed by removal of the t-butyl group gives 3-(2-carboxyethyl)-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, 3-(2-carboxy-ethyl)-4-(pyrid-2-aminocarbonyl)isoxazole, 3-(2-carboxyethyl)-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]isoxazole, and 3-(2-carboxyethyl)-[4-(trifluoromethylsulfonyl)phenyl-aminocarbonyl]isoxazole

u. 3-(2-Carboxyethyl)-4-[4-(trifluoromethyl)phenylamino-carbonyl]pyrazole

3-(2-carboxyethyl)-4-(pyrid-2-aminocarbonyl)pyrazole;

3-(2-Carboxyethyl)-4-[3-methyl-4-(trifluoromethyl) phenylaminocarbonyl]pyrazole;

3-(2-carboxyethyl)-[4-(trifluoromethylsulfonyl)-phenylaminocarbonyl]pyrazole.

By substituting ethyl 2-(t-butoxycarbonyl)priopiono-acetate for ethyl cyclopropylcarbonylacetate in g, and then substituting the appropriate amines for 4-(trifluoromethyl)-

aniline in g, the identical process, followed by removal of the t-butyl group, gives 3-(2-carboxyethyl)-4-[4-(trifluoromethyl)phenylaminocarbonyl]pyrazole, 3-(2-carboxyethyl)-4-(pyrid-2-aminocarbonyl)pyrazole, 3-(2-carboxyethyl)-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]pyrazole, and 3-(2-carboxyethyl)-[4-(trifluoromethylsulfonyl)-phenylaminocarbonyl]pyrazole.

v. 4-(1,3-Benzodioxan-6-aminocarbonyl)-3-methylisoxazole

4-(1,3-Benzodioxan-6-aminocarbonyl)-3-cyclopropylisoxazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-3,5-dimethylisoxazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-3-cyclopropyl-5-methylisoxazole;

4-(1,3-benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-methylisoxazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylisoxazole.

By substituting 6-amino-1,3-benzodioxane for 4-(trifluoromethyl)aniline in a, and then substituting the appropriate substituted priopiolates for methyl priopiolate and/or the appropriate substituted nitromethanes for nitroethane in a, the identical process gives 4-(1,3-benzodioxan-6-aminocarbonyl)-3-methylisoxazole, 4-(1,3-benzo-dioxan-6-aminocarbonyl)-3-cyclopropylisoxazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-3,5-dimethylisoxazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-3-cyclopropyl-5-methylisoxazole,4-(1,3-benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-methylisoxazole, and 4-(1,3-benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylisoxazole.

w. 4-(1,3-Benzodioxan-6-aminocarbonyl)-3-methylpyrazole

4-(1,3-Benzodioxan-6-aminocarbonyl)-3-cyclopropylpyrazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-3,5-dimethylpyrazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-3-cyclopropyl-5-methylpyrazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-methylpyrazole;

4-(1,3-Benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylpyrazole.

By substituting 6-amino-1, 3-benzodioxane for 4-(trifluoromethyl)aniline in g, and then the appropriate substituted acetoacetate for methyl cyclopropylcarbonyl-acetoacetate and/or the appropriate substituted orthoester for triethylorthoformate in the second step of b, the identical process of the second step of b and of g gives 4-(1,3-benzodioxan-6-aminocarbonyl)-3-methylpyrazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-3-cyclopropylpyrazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-3,5-dimethylpyrazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-3-cyclopropyl-5-methylpyrazole, 4-(1,3-benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-methylpyrazole, and 4-(1,3-benzodioxan-6-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylpyrazole.

x. 4-(1,3-Benzodioxol-5-aminocarbonyl)-3-methylisoxazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-3-cyclopropylisoxazole;

4-(1,3-Benzodioxol-5-aminocarbonyl])-3,5-dimethylisoxazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-3-cyclopropyl-5-methylisoxazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-methylisoxazole;

4-(1,3-benzo-dioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylisoxazole.

By substituting 5-amino-1,3-benzodioxole for 4-(trifluoromethyl)aniline in a, and then substituting the

appropriate substituted propiolic ester for ethyl propiolate and/or the appropriate stubstituted nitromethane for nitroethane in a, the identical process gives 4-(1,3-benzodioxol-5-aminocarbonyl)-3-methylisoxazole, 4-(1,3-benzodioxol-5-aminocarbonyl)-3-cyclopropylisoxazole, 4-(1,3-benzodioxol-5-aminocarbonyl])-3,5-dimethylisoxazole, 4-(1,3-benzodioxol-5-aminocarbonyl)-3-cyclopropyl-5-methylisoxazole, and 4-(1,3-benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-methylisoxazole, and 4-(1,3-benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylisoxazole.

y. 4-(1,3-Benzodioxol-5-aminocarbonyl)-3-methyl-pyrazole

4-(1,3-Benzodioxol-5-amino-carbonyl)-3-cyclopropyl-pyrazole;

4-(1,3-Benzodioxol-5-aminocarbonyl])-3,5-dimethyl-pyrazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-3-cyclopropyl-5-methylpyrazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-methylpyrazole;

4-(1,3-Benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylpyrazole.

By substituting 5-amino-1,3-benzodioxole for 4-(trifluoromethyl)aniline in g, and then substituting the appropriate substituted acetoacetate for ethyl cyclopropylcarbonylacetate and/or the appropriate substituted orthoformate for triethylorthoformate in the second step of b, the identical process of the second step of b and of g gives 4-(1,3-benzodioxol-5-aminocarbonyl)-3-methylpyrazole, 4-(1,3-benzodioxol-5-aminocarbonyl)-3-cyclopropylpyrazole, 4-(1,3-benzodioxol-5-aminocarbonyl])-3,5-dimethylpyrazole, 4-(1,3-benzodioxol-5-aminocarbonyl)-3-cyclopropyl-5-methylpyrazole, 4-(1,3-benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-methylpyrazole, and 4-(1,3-benzodioxol-5-aminocarbonyl)-5-(2-chlorophenyl)-3-cyclopropylpyrazole.

z. 3-Methyl-4-[4-(trifluoro- methyl)phenylamino-carbonyl]-thiazole

3-Methyl-4-(pyrid-2-aminocarbonyl)thiazole;

3-Methyl-4-[3-methyl-4-trifluoromethyl)phenylaminocarbonyl]thiazole;

3-Methyl-4-[4-(trifluoromethylsulfonyl)phenylamino-carbonyl]thiazole.

By substituting 3-methyl-4-thiazole carboxylic acid (Buttimore, D. et al.; J. Chem. Soc. (1963) p 2032 - 2039) for 3-methyl-4-isoxazole carboxylic acid in a, and then substituting the appropriate amines for 4-(trifluoromethyl) aniline in a, the identical process gives 3-methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]thiazole, 3-methyl-4-(pyrid-2-aminocarbonyl)thiazole, 3-methyl-4-[3-methyl-4-(trifluoromethyl)phenylaminocarbonyl]thiazole, and 3-methyl-4-[4-(trifluoromethylsulfonyl)phenylaminocarbonyl]thiazole.

Brief Description of the Tables

Table 1 is a comparison of the activity of MCTA, its metabolite and a compound of this invention as inhibitors of FGF induced DNA synthesis alone or with added uridine.

Table 2 shows the results of the ability of several of the compounds of this invention to inhibit DNA synthesis induced by FGF, PDGF and EGF.

Table 3 shows the results of a subcutaneous xenograft experiment testing the ability of Cmpd. 1 to inhibit tumor growth in vivo and, in addition, testing the toxic effects of Cmpd. 1 at the dose used.

Table 4 demonstrates that MCTA inhibits the specific biological response of cells to FGF while having no effect on the response of the cells to EGF. MCTA also inhibits the specific biological responses to PDGF. The results are shown as IC50s, the concentration at which 50% of the maximal BrdU incorporation response is inhibited.

Table 5 shows the results of subcutaneous xenograft experiments wherein administration of MCTA significantly inhibited the growth of tumors associated with FGFR activity with no significant effect on a non-FGFR expressing tumor thus demonstrating MCTA's effectiveness as a treatment for cancers characterized by inappropriate FGFR activity.

Brief Description of the Figures

Figure 1 graphically depicts the effect over time of 20, 10, 5.0 and 2.5 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone on tumor weight compared to that in the untreated controls.

Figure 2 is a graph of the effect over time of 27, 18, 12 and 8.0 mg/kg BCNU alone on tumor weight compared to the untreated control.

Figure 3 is a graphical comparison of the effect over time of 10.0 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone, 18 mg/kg BCNU alone and the combination of 10.0 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide with 18 mg/kg BCNU, administered as described in Section 4, below, compared to the control.

Figure 4 is a graph of the effect over time on tumor weight of 10 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone, 12 mg/kg BCNU alone and the combination of 10 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide with 12 mg/kg BCNU, again, compared to the untreated control.

Figure 5 graphically compares the effect over time on tumor weight of 5 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone, 18 mg/kg BCNU alone, the combination, in the manner described in Section 4, below, of 5 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide with 18 mg/kg BCNU and the untreated control.

Figure 6 is a graph depicting the effect over time on tumor weight of 5.0 mg/kg N-(4-trifluoromethylphenyl)-5-

methylisoxazole-4-carboxamide alone, 12 mg/kg BCNU alone, the combination of 5.0 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide with 12 mg/kg BCNU, compared as before with the untreated control.

Biological Evaluation Methods

It will be appreciated that, in any given series of compounds, a spectrum of biological activity will be afforded. In its most preferred embodiments, this invention relates to novel heteroarylcarboxamides demonstrating the ability to modulate PTK activity. The following assays are employed to select those compounds demonstrating the optimal degree of the desired activity.

As used herein, the phrase "optimal degree of the desired activity" refers to the lowest IC50, defined elsewhere herein, against a PTK related to a particular disorder so as to provide an organism, preferably a human, with a therapeutically effective amount of a compound of this invention at the lowest possible dosage.

The following *in vitro* assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the RTKs. Similar assays can be designed along the same lines for any PTK using techniques well known in the art.

The cellular/catalytic assays described herein are performed in an ELISA format. The general procedure is a follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor's activity is added. The cells are lysed and the lysate is transferred to the wells of an ELISA plate previously coated with a specific antibody recognizing the substrate of the enzymatic phosphorylation reaction. Non-substrate components of the cell lysate are washed away and the amount of phosphorylation on the substrate

is detected with an antibody specifically recognizing phosphotyrosine compared with control cells that were not contacted with a test compound.

The cellular/biologic assays described herein measure the amount of DNA made in response to activation of a test kinase, which is a general measure of a proliferative response. The general procedure for this assay is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor's activity is added. After incubation at least overnight, a DNA labeling reagent such as Bromodeoxy-uridine (BrdU) or 3H-thymidine is added. The amount of labeled DNA is detected with either an anti-BrdU antibody or by measuring radioactivity and is compared to control cells not contacted with a test compound.

1. Cellular/Catalytic Assays

Enzyme linked immunosorbent assays (ELISA) may be used to detect and measure the presence of PTK activity. The ELISA may be conducted according to known protocols which are described in, for example, Voller, et al., 1980, "Enzyme-Linked Immunosorbent Assay," In: Manual of Clinical Immunology, 2d ed., edited by Rose and Friedman, pp 359-371 Am. Soc. Of Microbiology, Washington, D.C.

The disclosed protocol may be adapted for determining activity with respect to a specific RTK. For example, the preferred protocols for conducting the ELISA experiments for specific RTKs is provided below. Adaptation of these protocols for determining a compound's activity for other members of the RTK family, as well as for CTKs, is well within the scope of knowledge of those skilled in the art.

a. FLK-1

An ELISA assay can be conducted to measure the kinase activity of the FLK-1 receptor and more specifically, the inhibition or activation of TK activity on the FLK-1 receptor. Specifically, the following assay can be conducted to measure kinase activity of the FLK-1 receptor in cells genetically engineered to express Flk-1.

Materials And Methods

Materials. The following reagents and supplies are being used:

a. Corning 96-well ELISA plates (Corning Catalog No. 25805-96);
b. Cappel goat anti-rabbit IgG (catalog no. 55641);
c. PBS (Gibco Catalog No. 450-1400EB);
d. TBSW Buffer (50 mM Tris (pH 7.2), 150 mM NaCl and 0.1% Tween-20);
e. Ethanolamine stock (10% ethanolamine (pH 7.0), stored at 4°C);
f. HNTG buffer (20mM HEPES buffer (pH 7.5), 150mM NaCl, 0.2% Triton X-100, and 10% glycerol);
g. EDTA (0.5 M (pH 7.0) as a 100X stock);
h. Sodium ortho vanadate (0.5 M as a 100X stock);
i. Sodium pyro phosphate (0.2M as a 100X stock);
j. NUNC 96 well V bottom polypropylene plates (Applied Scientific Catalog No. AS-72092);
k. NIH3T3 C7#3 Cells (FLK-1 expressing cells);
l. DMEM with 1X high glucose L Glutamine (catalog No. 11965-050);
m. FBS, Gibco (catalog no. 16000-028);
n. L-glutamine, Gibco (catalog no. 25030-016);
o. VEGF, PeproTech, Inc. (catalog no. 100-20)(kept as 1 µg/100µl stock in Milli-Q dH_2O and stored at -20°C;
p. Affinity purified anti-FLK-1 antiserum;

q. UB40 monoclonal antibody specific for phosphotyrosine (*see*, Fendley, *et al.*, 1990, *Cancer Research* 50:1550-1558);

r. EIA grade Goat anti-mouse IgG-POD (BioRad catalog no. 172-1011);

s. 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (100mM citric acid (anhydrous), 250 mM Na_2HPO_4 (pH 4.0), 0.5 mg/ml ABTS (Sigma catalog no. A-1888)), solution should be stored in dark at 4°C until ready for use;

t. H_2O_2 (30% solution)(Fisher catalog no. H325);

u. ABTS/H_2O_2 (15ml ABTS solution, 2 µl H_2O_2) prepared 5 minutes before use and left at room temperature;

v. 0.2 M HCl stock in H_2O;

w. dimethylsulfoxide (100%)(Sigma Catalog No. D-8418); and

y. Trypsin-EDTA (Gibco BRL Catalog No. 25200-049).

Protocol. The following protocol is being used for conducting the assay:

1. Coat Corning 96-well elisa plates with 1.0µg per well Cappel Anti-rabbit IgG antibody in 0.1M Na_2CO_3 pH 9.6. Bring final volume to 150 µl per well. Coat plates overnight at 4°C. Plates can be kept up to two weeks when stored at 4°C.

2. Grow cells in Growth media(DMEM, supplemental with 2.0mM L-Glutamine, 10% FBS) in suitable culture dishes until confluent at 37°C, 5% CO_2.

3. Harvest cells by trypsinization and seed in Corning 25850 polystyrene 96-well roundbottom cell plates, 25.000 cells/well in 200µl of growth media.

4. Grow cells at least one day at 37°C, 5% CO_2.

5. Wash cells with D-PBS 1X.

6. Add 200µl/well of starvation media (DMEM, 2.0mM 1-Glutamine, 0.1% FBS). Incubate overnight at 37°C, 5% CO_2.

7. Dilute Compounds 1:20 in polypropylene 96 well plates using starvation media. Dilute dimethylsulfoxide 1:20 for use in control wells.

8. Remove starvation media from 96 well cell culture plates and add 162 µl of fresh starvation media to each well.

9. Add 18µl of 1:20 diluted Compound dilution (from step 7) to each well plus the 1:20 dimethylsulfoxide dilution to the control wells (+/- VEGF), for a final dilution of 1:200 after cell stimulation. Final dimethylsulfoxide is 0.5%. Incubate the plate at 37°C, 5% CO_2 for two hours.

10. Remove unbound antibody from ELISA plates by inverting plate to remove liquid. Wash 3 times with TBSW + 0.5% ethanolamine, pH 7.0. Pat the plate on a paper towel to remove excess liquid and bubbles.

11. Block plates with TBSW + 0.5% Ethanolamine, pH 7.0, 150 µl per well. Incubate plate thirty minutes while shaking on a microtiter plate shaker.

12. Wash plate 3 times as described in step 10.

13. Add 0.5µg/well affinity purified anti-FLU-1 polyclonal rabbit antiserum. Bring final volume to 150µl/well with TBSW + 0.5% ethanolamine pH 7.0. Incubate plate for thirty minutes while shaking.

14. Add 180 µl starvation medium to the cells and stimulate cells with 20µl/well 10.0mM sodium orthovanadate and 500 ng/ml VEGF (resulting in a final concentration of 1.0mM sodium orthovanadate and 50ng/ml VEGF per well) for eight minutes at 37°C, 5% CO_2. Negative control wells receive only starvation medium.

15. After eight minutes, media should be removed from the cells and washed one time with 200µl/well PBS.

16. Lyse cells in 150µl/well HNTG while shaking at room temperature for five minutes. HNTG formulation includes sodium orthovanadate, sodium pyrophosphate and EDTA.

17. Wash ELISA plate three times as described in step 10.

18. Transfer cell lysates from the cell plate to ELISA plate and incubate while shaking for two hours. To transfer cell lysate pipette up and down while scrapping the wells.

19. Wash plate three times as described in step 10.

20. Incubate ELISA plate with 0.02µg/well UB40 in TBSW + 0.5% ethanolamine. Bring final volume to 150µl/well. Incubate while shaking for 30 minutes.

21. Wash plate three times as described in step 10.

22. Incubate ELISA plate with 1:10,000 diluted EIA grade goat anti-mouse IgG conjugated horseradish peroxidase in TBSW + 0.5% ethanolamine, pH 7.0. Bring final volume to 150µl/well. Incubate while shaking for thirty minutes.

23. Wash plate as described in step 10.

24. Add 100 µl of ABTS/H_2O_2 solution to well. Incubate ten minutes while shaking.

25. Add 100 µl of 0.2 M HCl for 0.1 M HCl final to stop the color development reaction. Shake 1 minute at room temperature. Remove bubbles with slow stream of air and read the ELISA plate in an ELISA plate reader at 410 nm.

b. HER-2 ELISA

Assay 1: EGF Receptor-HER2 Chimeric Receptor Assay In Whole Cells.

HER2 kinase activity in whole EGFR-NIH3T3 cells are measured as described below:

Materials and Reagents. The following materials and reagents are being used to conduct the assay:

a. EGF: stock concentration = 16.5 ILM; EGF 201, TOYOBO, Co., Ltd. Japan.

b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

c. Anti-phosphotyrosine antibody (anti-Ptyr) (polyclonal) (*see*, Fendley, *et al.*, *supra*).

d. Detection antibody: Goat anti-rabbit lgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.

e. TBST buffer:

Tris-HCl, pH 7.2	50 mM
NaCl	150 mM
Triton X-100	0.1

f. HNTG 5X stock:

HEPES	0.1 M
NaCl	0.75 M
Glycerol	50%
Triton X-100	1.0%

g. ABTS stock:

Citric Acid	100 mM
Na_2HPO_4	250 mM
HCl, conc.	0.5 pM
ABTS*	0.5mg/ml

*2,2'azinobis(3-ethylbenzthiazolinesulfonic acid).

Keep solution in dark at 4°C until use.

h. Stock reagents of:

EDTA 100 mM pH 7.0

Na_3VO_4 0.5 M

$Na_4(P_2O_7)$ 0.2 M

Procedure. The following protocol is being used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody at 0.5 g per well in PBS, 100 µl final volume/well, and store overnight at 4°C. Coated plates are good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and replace with 100 µl blocking buffer (5% Carnation Instant Non-Fat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to use, remove blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. An NIH3T3 cell line overexpressing a chimeric receptor containing the EGFR extracellular domain and intracellular HER2 kinase domain can be used for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% fetal bovine serum. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1500 rpm, at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 µl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37°C for about 40 hours.

C. Assay Procedures

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 µl to a TBST well for a final drug dilution of 1:200 and a final DMSO concentration of 1%. Control wells receive DMSO alone. Incubate in 5% CO_2 at 37°C for two hours.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 µl dilute EGF (1:12 dilution), 100 nM final concentration is attained.

3. Prepare fresh HNTG* sufficient for 100 µl per well; and place on ice.

HNTG* (10 ml):

HNTG stock	2.0 ml
milli-Q H_2O	7.3 ml
EDTA, 100 mM, pH 7.0	0.5 ml
Na_3VO_4, 0.5 M	0.1 ml
$Na_4(P_2O_7)$, 0.2 M	0.1 ml

4. After 120 minutes incubation with drug, add prepared SGF ligand to cells, 10 µl per well, to a final concentration of 100 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

5. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 µl per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

6. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

7. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 µl per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

8. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO anti-rabbit IgG antibody to the ELISA plate at 100 µl per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

9. Remove TAGO detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 µl per well. Incubate shaking at room temperature for 20 minutes. (ABTS/H_2O_2 solution: 1.0 µl 30% H_2O_2 in 10 ml ABTS stock).

10. Stop reaction by adding 50 µl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

11. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

c. PDGF-R ELISA

All cell culture media, glutamine, and fetal bovine serum can be purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

For ELISA assays, cells (U1242, obtained from Joseph Schlessinger, NYU) are grown to 80-90% confluency in growth medium (MEM with 10% FBS, NEAA, 1 mM NaPyr and 2 mM GLN) and seeded in 96-well tissue culture plates in 0.5% serum at 25,000 to 30,000 cells per well. After overnight incubation in 0.5% serum-containing medium, cells are changed to serum-free medium and treated with test compound for 2 hr in a 5% CO_2, 37°C incubator. Cells are then stimulated with ligand for 5-10 minute followed by lysis with HNTG (20 mM Hepes, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM Na_3VO_4, 0.2% Triton X-100, and 2 mM NaPyr). Cell lysates (0.5 mg/well in PBS) are transferred to ELISA plates previously coated with receptor-specific antibody and which had been blocked with 5% milk in TBST (50 mM Tris-HCl pH 7.2, 150 mM NaCl and 0.1% Triton X-100) at room temperature for 30 min. Lysates are incubated with shaking for 1 hour at room temperature. The plates are washed with TBST four times and then incubated with polyclonal anti-phosphotyrosine antibody at room temperature for 30 minutes. Excess anti-phosphotyrosine antibody was removed by rinsing the plate with TBST four times. Goat anti-rabbit IgG antibody was added to the ELISA plate for 30 min at room temperature followed by rinsing with TBST four more times. ABTS (100 mM citric acid, 250 mM Na_2HPO_4 and 0.5 mg/ml 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) plus H_2O_2 (1.2 ml 30% H_2O_2 to 10 ml ABTS) was added to the ELISA plates to start color development. Absorbance at 410 nm with a reference wavelength of 630 nm was recorded about 15 to 30 min after ABTS addition.

d. IGF-I Receptor ELISA

The following protocol may be used to measure phosphotyrosine level on IGF-I receptor, which indicates IGF-I receptor tyrosine kinase activity.

Materials And Reagents. The following materials and reagents are used:

a. The cell line used in this assay is 3T3/IGF-1R, a cell line genetically engineered to overexpresses IGF-1 receptor.

b. NIH3T3/IGF-1R is grown in an incubator with 5% CO_2 at 37°C. The growth media is DMEM + 10% FBS (heat inactivated)+ 2mM L-glutamine.

c. Affinity purified anti-IGF-1R antibody 17-69.

d. D-PBS:

KH_2PO_4	0.20 g/l
K_2HPO_4	2.16 g/l
KCl	0.20 g/l
NaCl	8.00 g/l (pH 7.2)

e. Blocking Buffer: TBST plus 5% Milk (Carnation Instant Non-Fat Dry Milk).

f. TBST buffer:

Tris-HCl	50 mM
NaCl	150mM (pH 7.2/HCl 10N)
Triton X-100	0.1%

Stock solution of TBS (10X) is prepared, and Triton X-100 is added to the buffer during dilution.

g. HNTG buffer:

HEPES	20 mM
NaCl	150 mM (pH 7.2/HCl 1N)
Glycerol	10%
Triton X-100	0.2%

Stock solution (5X) is prepared and kept at 4°C.

h. EDTA/HCl: 0.5 M pH 7.0 (NaOH) as 100X stock.

i. Na_3VO_4: 0.5 M as 100X stock and aliquots are kept in -80°C.

j. $Na_4P_2O_7$: 0.2 M as 100X stock.

k. Insulin-like growth factor-1 from Promega (Cat# G5111).

l. Rabbit polyclonal anti-phosphotyrosine antiserum.

m. Goat anti-rabbit IgG, POD conjugate (detection antibody), Tago (Cat. No. 4520, Lot No. 1802): Tago, Inc., Burlingame, CA.

n. ABTS (2.2'-azinobis(3-ethylbenzthiazolinesulfonic acid)) solution:

Citric acid	100 mM
Na_2HPO_4	250 mM (pH 4.0/1 N HCl)
ABTS	0.5 mg/ml

ABTS solution should be kept in dark and 4°C. The solution should be discarded when it turns green.

o. Hydrogen Peroxide: 30% solution is kept in the dark and at 4°C.

Procedure. All the following steps are conducted at room temperature unless it is specifically indicated. All ELISA plate washings are performed by rinsing the plate with tap water three times, followed by one TBST rinse. Pat plate dry with paper towels.

A. Cell Seeding:

1. The cells, grown in tissue culture dish (Corning 25020-100) to 80-90% confluence, are harvested with Trypsin-EDTA (0.25%, 0.5 ml/D-100, GIBCO).

2. Resuspend the cells in fresh DMEM + 10% FBS + 2mM L-Glutamine, and transfer to 96-well tissue culture plate (Corning, 25806-96) at 20,000 cells/well (100 µl/well). Incubate for 1 day then replace medium to serum-free medium (90/µl) and incubate in 5% CO_2 and 37°C overnight.

B. ELISA Plate Coating and Blocking:

1. Coat the ELISA plate (Corning 25805-96) with Anti-IGF-1R Antibody at 0.5 µg/well in 100 µl PBS at least 2 hours.

2. Remove the coating solution, and replace with 100 µl Blocking Buffer, and shake for 30 minutes. Remove the blocking buffer and wash the plate just before adding lysate.

C. Assay Procedures:

1. The drugs are tested in serum-free condition.

2. Dilute drug stock (in 100% DMSO) 1:10 with DMEM in 96-well polypropylene plate, and transfer 10 µl/well of this solution to the cells to achieve final drug dilution 1:100, and final DMSO concentration of 1.0%. Incubate the cells in 5% CO_2 at 37°C for 2 hours.

3. Prepare fresh cell lysis buffer (HNTG*)

HNTG	2 ml
EDTA	0.1 ml
Na_3VO_4	0.1 ml
$Na_4(P_2O_7)$	0.1 ml
H_2O	7.3 ml

4. After drug incubation for two hours, transfer 10 µl/well of 200nM IGF-1 Ligand in PBS to the cells (Final Conc. = 20 nM), and incubate at 5% CO_2 at 37°C for 10 minutes.

5. Remove media and add 100µl/well HNTG* and shake for 10 minutes. Look at cells under microscope to see if they are adequately lysed.

6. Use a 12-channel pipette to scrape the cells from the plate, and homogenize the lysate by repeat aspiration and dispense. Transfer all the lysate to the antibody coated ELISA plate, and shake for 1 hour.

7. Remove the lysate, wash the plate, transfer anti-pTyr (1:3,000 with TBST) 100 µl/well, and shake for 30 minutes.

8. Remove anti-pTyr, wash the plate, transfer Tago (1:3,000 with TBST) 100 µl/well, and shake for 30 minutes.

9. Remove detection antibody, wash the plate, and transfer fresh ABTS/H2O2 (1.2 µl H_2O_2 to 10 ml ABTS) 100 µl/well to the plate to start color development.

10. Measure OD at 410 nm with a reference wavelength of 630 nm in Dynatec MR5000.

e. EGF Receptor ELISA

EGF Receptor kinase activity in cells genetically engineered to express human EGF-R is measured as described below:

Materials and Reagents. The following materials and reagents are used

a. EGF Ligand: stock concentration = 16.5 μM; EGF 201, TOYOBO, Co., Ltd. Japan.

b. 05-101 (UBI) (a monoclonal antibody recognizing an EGFR extracellular domain).

c. Anti-phosphotyosine antibody (anti-Ptyr)(polyclonal).

d. Detection antibody: Goat anti-rabbit IgG horse radish peroxidase conjugate, TAGO, Inc., Burlingame, CA.

e. TBST buffer:

Tris-HCl, pH 7	50 mM
NaCl	150 mM
Triton X-100	0.1

f. HNTG 5X stock:

HEPES	0.1 M
NaCl	0.75 M
Glycerol	50
Triton X-100	1.0%

g. ABTS stock:

Citric Acid	100 mM
Na_2HPO_4	250 mM
HCl, conc.	4.0 pH
ABTS*	0.5 mg/ml

Keep solution in dark at 4°C until used.

h. Stock reagents of:

EDTA 100 mM pH 7.0

Na_3VO_4 0.5 M

$Na_4(P_2O_7)$ 0.2 M

Procedure. The following protocol was used:

A. Pre-coat ELISA Plate

1. Coat ELISA plates (Corning, 96 well, Cat. #25805-96) with 05-101 antibody at 0.5 µg per well in PBS, 150 µl final volume/well, and store overnight at 4°C. Coated plates are good for up to 10 days when stored at 4°C.

2. On day of use, remove coating buffer and replace with blocking buffer (5% Carnation Instant NonFat Dry Milk in PBS). Incubate the plate, shaking, at room temperature (about 23°C to 25°C) for 30 minutes. Just prior to use, remove blocking buffer and wash plate 4 times with TBST buffer.

B. Seeding Cells

1. NIH 3T3/C7 cell line (Honegger, et al., Cell 51:199-209, 1987) can be use for this assay.

2. Choose dishes having 80-90% confluence for the experiment. Trypsinize cells and stop reaction by adding 10% CS DMEM medium. Suspend cells in DMEM medium (10% CS DMEM medium) and centrifuge once at 1000 rpm, and once at room temperature for 5 minutes.

3. Resuspend cells in seeding medium (DMEM, 0.5% bovine serum), and count the cells using trypan blue. Viability above 90% is acceptable. Seed cells in DMEM medium (0.5% bovine serum) at a density of 10,000 cells per well, 100 µl per well, in a 96 well microtiter plate. Incubate seeded cells in 5% CO_2 at 37°C for about 40 hours.

C. Assay Procedures.

1. Check seeded cells for contamination using an inverted microscope. Dilute drug stock (10 mg/ml in DMSO) 1:10 in DMEM medium, then transfer 5 µl to a test well for a final drug dilution of 1:200 and a final DMSO concentration of 1%.

Control wells receive DMSO alone. Incubate in 5% CO_2 at 37°C for one hour.

2. Prepare EGF ligand: dilute stock EGF in DMEM so that upon transfer of 10 µl dilute EGF (1:12 dilution), 25 nM final concentration is attained.

3. Prepare fresh 10 ml HNTG* sufficient for 100 µl per well wherein HNTG* comprises: HNTG stock (2.0 ml), milli-Q H_2O (7.3 ml), EDTA, 100 mM, pH 7.0 (0.5 ml), Na_3VO_4 0.5 M (0.1 ml) and $Na_4(P_2O_7)$, 0.2 M (0.1 ml).

4. Place on ice.

5. After two hours incubation with drug, add prepared EGF ligand to cells, 10 µl per well, to yield a final concentration of 25 nM. Control wells receive DMEM alone. Incubate, shaking, at room temperature, for 5 minutes.

6. Remove drug, EGF, and DMEM. Wash cells twice with PBS. Transfer HNTG* to cells, 100 µl per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from other ELISA plate and wash with TBST as described above.

7. With a pipette tip securely fitted to a micropipettor, scrape cells from plate and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, and washed ELISA plate. Incubate shaking at room temperature for one hour.

8. Remove lysate and wash 4 times with TBST. Transfer freshly diluted anti-Ptyr antibody to ELISA plate at 100 µl per well. Incubate shaking at room temperature for 30 minutes in the presence of the anti-Ptyr antiserum (1:3000 dilution in TBST).

9. Remove the anti-Ptyr antibody and wash 4 times with TBST. Transfer the freshly diluted TAGO 30 anti-rabbit IgG antibody to the ELISA plate at 100 µl per well. Incubate shaking at room temperature for 30 minutes (anti-rabbit IgG antibody: 1:3000 dilution in TBST).

10. Remove detection antibody and wash 4 times with TBST. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA

plate, 100 µl per well. Incubate at room temperature for 20 minutes. ABTS/H_2O_2 solution: 1.2 µl 30% H_2O_2 in 10 ml ABTS stock.

11. Stop reaction by adding 50 µl 5N H_2SO_4 (optional), and determine O.D. at 410 nm.

12. The maximal phosphotyrosine signal is determined by subtracting the value of the negative controls from the positive controls. The percent inhibition of phosphotyrosine content for extract-containing wells is then calculated, after subtraction of the negative controls.

2. Cellular/Biologic Assays

Assay 1: PDGF-Induced BrdU Incorporation Assay

Materials and Reagents:

(1) PDGF: human PDGF B/B; 1276-956, Boehringer Mannheim, Germany

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) 3T3 cell line genetically engineered to express human PDGF-R.

Protocol

(1) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (PDGF=3.8 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (PDGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 2: EGF-Induced BrdU Incorporation Assay

Materials and Reagents

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) 3T3 cell line genetically engineered to express human EGF-R.

Protocol

(1) Cells are seeded at 8000 cells/well in 10% CS, 2mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (EGF=2 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 3: EGF-Induced Her2-Driven BrdU Incorporation

Materials and Reagents:

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) 3T3 cell line engineered to express a chimeric receptor having the extra-cellular domain of EGF-R and the intra-cellular domain of Her2.

Protocol:

(1) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96- well plate. Cells are incubated overnight at 37° in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (EGF=2 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (EGF) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Assay 4: IGF1-Induced BrdU Incorporation Assay

Materials and Reagents:

(1) IGF1 Ligand: human, recombinant; G511, Promega Corp, USA.

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution: 1X PBS, pH 7.4, made in house.

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) 3T3 cell line genetically engineered to express human IGF-1 receptor.

Protocol:

(1) Cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96-well plate. Cells are incubated overnight at 37°C in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then are serum starved in serum free medium (0% CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (IGF1=3.3 nM, prepared in DMEM with 0.1% BSA) and test compounds are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand (IGF1) but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 16 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

c. HUV-EC-C Assay

The following protocol may also be used to measure a compound's activity against PDGF-R, FGF-R or Flk-1/KDR, all of which are naturally expressed by HUV-EC cells.

Day 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, (American Type Culture Collection; catalogue no. 1730 CRL). Wash with Dulbecco's phosphate-buffered saline (D-PBS; obtained from Gibco BRL; catalogue no. 14190-029) 2 times at about 1 ml/10 cm^2 of tissue culture flask. Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company; catalogue no. C-1544). The 0.05% trypsin was made by diluting 0.25% trypsin/1 mM EDTA (Gibco; catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 ml/25-30 cm^2 of tissue culture flask for about 5 minutes at 37°C. After cells have detached from the flask, add an equal volume of assay medium and transfer to a 50 ml sterile centrifuge tube (Fisher Scientific; catalogue no. 05-539-6).

2. Wash the cells with about 35 ml assay medium in the 50 ml sterile centrifuge tube by adding the assay medium,

centrifuge for 10 minutes at approximately 200xg, aspirate the supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two more times with D-PBS, resuspend the cells in about 1 ml assay medium/15 cm^2 of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL; catalogue no. 21127-014) + 0.5% heat-inactivated fetal bovine serum. Count the cells with a Coulter Counter®v Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of 0.8-1.0x10^5 cells/ml.

3. Add cells to 96-well flat-bottom plates at 100 µl/well or 0.8-1.0x10^4 cells/well; incubate ~24h at 37°C, 5% CO_2.

Day 1

1. Make up two-fold drug titrations in separate 96-well plates, generally 50 µM on down to 0 µM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 µl/well of drug at 200 µM (4X the final well concentration) to the top well of a particular plate column. Since the stock drug concentration is usually 20 mM in DMSO, the 200 µM drug concentration contains 2% DMSO.

Therefore, diluent made up to 2% DMSO in assay medium (F12K + 0.5% fetal bovine serum) is used as diluent for the drug titrations in order to dilute the drug but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 µl/well. Take 60 µl from the 120 µl of 200 µM drug dilution in the top well of the column and mix with the 60 µl in the second well of the column. Take 60 µl from this well and mix with the 60 µl in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 µl of the 120 µl in this well and discard it. Leave the last well with 60 µl of DMSO/media diluent as a non-drug-containing control. Make 9 columns of titrated drug, enough for triplicate wells each for 1) VEGF (obtained from Pepro Tech Inc., catalogue no.

100-200, 2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained from Boehringer Mannheim Biochemica, catalogue no. 1439 600); or, 3) human PDGF B/B (1276-956, Boehringer Mannheim, Germany) and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 µl/well of the drug dilutions to the 96-well assay plates containing the $0.8-1.0x10^4$ cells/100 µl/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37°C, 5% CO_2.

3. In triplicate, add 50 µl/well of 80 µg/ml VEGF, 20 ng/ml ECGF, or media control to each drug condition. As with the drugs, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24 hours at 37°C, 5% CO_2. Each well will have 50 µl drug dilution, 50 µl growth factor or media, and 100 ul cells, = 200 ul/well total. Thus the 4X concentrations of drugs and growth factors become 1X once everything has been added to the wells.

Day 2

1. Add ^{3}H-thymidine (Amersham; catalogue no. TRK-686) at 1 µCi/well (10 µl/well of 100 µCi/ml solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37°C, 5% CO_2. RPMI was obtained from Gibco BRL, catalogue no. 11875-051.

Day 3

1. Freeze plates overnight at -20°C.

Day 4

1. Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96$^{(R)}$) onto filter mats (Wallac;

catalogue no. 1205-401); read counts on a Wallac Betaplate(TM) liquid scintillation counter.

C. *In Vivo* Animal Models

1. Xenograft Animal Models

The ability of human tumors to grow as xenografts in athymic mice (*e.g.*, Balb/c, nu/nu) provides a useful in vivo model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, Acta Pathol. Microbial. Scand. 77:758-760), many different human tumor cell lines (*e.g.*, mammary, lung, genitourinary, gastro-intestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. The following assays may be used to determine the level of activity, specificity and effect of the different compounds of the present invention. Three general types of assays are useful for evaluating compounds: cellular/catalytic, cellular/biological and in vivo. The object of the cellular/catalytic assays is to determine the effect of a compound on the ability of a TK to phosphorylate tyrosines on a known substrate in a cell. The object of the cellular/biological assays is to determine the effect of a compound on the biological response stimulated by a TK in a cell. The object of the in vivo assay is to determine the effect of a compound in an animal model of a particular disorder such as cancer.

Suitable cell lines for subcutaneous xenograft experiments include C6 cells (glioma, ATCC # CCL 107), A375 cells (melanoma, ATCC # CRL 1619), A431 cells (epidermoid carcinoma, ATCC # CRL 1555), Calu 6 cells (lung, ATCC # HTB 56), PC3 cells (prostate, ATCC # CRL 1435) and NIH 3T3 fibroblasts genetically engineered to overexpress EGFR, PDGFR, IGF-1R or any other test kinase.

The following protocol can be used to perform xenograft experiments:

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They receive sterile rodent chow and water ad libitum.

Cell lines are grown in appropriate medium (for example, MEM, DMEM, Ham's F10, or Ham's F12 plus 5% - 10% fetal bovine serum (FBS) and 2 mM glutamine (GLN)). All cell culture media, glutamine, and fetal bovine serum are purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, 2 - 10 x 10^6 cells/animal). Tumor growth is measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students' t-test. Test compounds in 50 - 100 µL excipient (DMSO, or VPD:D5W) was delivered by IP injection at different concentrations generally starting at day one after implantation.

2. Tumor Invasion Model

The following tumor invasion model has been developed and maybe used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor.

Procedure

8 week old nude mice (female) (Simonsen Inc.) are used as experimental animals. Implantation of tumor cells was performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10^7 tumor cells in a volume of 100 µl medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin was closed by using would clips. Animals are observed daily.

Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurement of tumor size, grade of invasion, immunochemistry and in situ hybridization).

7. EXAMPLES

A. Compounds of this invention.

The following are examples of the results of specific assays used to evaluate the activity of the compounds of this invention. The assays shown are exemplary only and are not to be construed as limiting in any manner.

1. Inhibition of Ligand-Stimulated DNA Synthesis.

The following example illustrates the ability of the compounds of the invention to inhibit FGFR-stimulated and PDGFR-stimulated DNA synthesis in cells. DNA synthesis is required for many of the activities of FGFR and PDGFR

including, but not limited to, cell proliferation. Uridine is added in one set of samples to overcome any contribution made by inhibition of DHOD and just evaluate the inhibition of PDGFR or FGFR signaling. (See Greene, et al., Biochem. Pharmacol., 50(6):861 (1995), Nair, et al., Immunology Letters, 47:171 (1995)).

Materials And Methods

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan; PDGF, Boehringer Mannheim, Germany; FGF, Gibco.

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4),Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X phosphate buffered saline, pH 7.4

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) NIH3T3 clone C7 (3T3/EGFRc7)(Honegger et al., Cell 51:199-209, 1987)) engineered to over-express human EGF receptor. These cells natively express FGFR and PDGFR.

Protocol

(1) 3T3/EGFRc7 cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37° C in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then serum starved in serum free medium (0%CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (2nM EGF or 1.5 nM FGF or 3.8 nM PDGF) prepared in DMEM with 0.1% BSA and 30 µM (final concentration) uridine) and test compound is added to the cells simultaneously. The negative control wells received serum free DMEM with 0.1% BSA only; the positive control cells received ligand but no test compound. Test compound is prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate was incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) was added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS,

and the plate dried by inverting and tapping on a paper towel.

(9) TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development was sufficient for photometric detection.

(10) The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Results

In a first experiment, the activity of MCTA, its metabolite and a compound of this invention are compared in their ability to inhibit FGF induced DNA synthesis alone or with added uridine. As shown in Table 1 below, the ability of the metabolite to inhibit DNA synthesis is completely abolished by the addition of uridine and that of MCTA reduced, demonstrating that the inhibitory effect is due to inhibition of DHOD, not inhibition of FGFR signaling. In contrast, the inhibitory activity of claimed compound is not decreased, and is even slightly increased, demonstrating that it is inhibiting FGFR signaling. Similar experiments are conducted using PDGF stimulation with similar results. Also tested was the known DHOD inhibitor brequinar which inhibited DNA synthesis in the absence of uridine (IC50 = 1.6 µM) and was inactive in the presence of uridine (IC50 = >100µM).

Table 1

Compound	IC50 - uridine	IC50 - uridine
MCTA	20 μM	80 μM
A771726	20 μM	>100μM
3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole (cmpd. 1)		

In another experiment, several of the compounds of this invention are tested for their ability to inhibit DNA synthesis induced by FGF, PDGF and EGF. The results, shown in Table 2 below, indicate that the compounds of the invention are selective for inhibition of FGF and PDGF induced signaling compared to EGF signaling.

Table 2

Compound	PDGF-induced	FGF-induced	EGF-induced
3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]pyrazole (cmpd. 2)	95 μM	>100 μM	>100 μM
3,5-Dimethyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole (cmpd. 21)	>100 μM	>100 μM	>100 μM
3-Methyl-4-[4-(trifluoromethyl)phenylaminocarbonyl]isoxazole (cmpd. 1)	90 μM	65 μM	>100 μM

2. Inhibition of Tumor Growth in vivo

The following example demonstrates the ability of Cmpd. 1 to inhibit the in vivo growth of tumors characterized by

inappropriate FGFR and/or PDGFR activity. The FGFR expressing cells are from two glioblastomas (C6, ATCC CRL 107, Powell and Klagsbrun, Exp. Cell Res., 209:224 (1993); for PDGFR, see Strawn, et al., J. Biol. Chem., 269:21215 (1995).

Materials And Methods

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They received sterile rodent chow and water ad libitum.

Cell lines are grown in Ham's F10 plus 5% fetal bovine serum (FBS) and 2 mM glutamine (GLN). All cell culture media, glutamine, and fetal bovine serum is purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, 3 x 10^6 cells/animal). Tumor growth was measured over 3 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students' t-test. Cmpd. 1 in 50 µL excipient (DMSO) was delivered by IP bolus injection daily.

Results

The results of the subcutaneous xenograft experiment, shown in Table 3 below, demonstrates that administration of Cmpd. 1 significantly inhibited the tumor growth in vivo and had no toxic effect at the dose tested.

Table 3

Treatment	Day	inhibition % of control)	mortality	p-value
DMSO alone	20	--	0	--
30 mg/kg/day (cmpd. 1)	8	34	0	0.0158
	10	42	0	0.0034
	14	48	0	0.0520
	15	47	0	0.0392
	17	50	0	0.0188
	20	52	0	0.0064

3. Inhibition of Tumor Growth and Metastasis in vivo.

The following example can be used to test the ability of the compounds of the invention to inhibit growth and metastasis of a tumor cell line expressing FGFR and PDGFR (C6 cells).

Materials And Methods

Ten to 12 week old athymic Balb/c nu/nu mice are obtained from Simonsen Laboratory (Gilroy, CA) and maintained in a pathogen-free environment throughout the experiments.

C6 cells (ATCC CCL 107) are grown and maintained in F-10 medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal bovine serum, 2mM glutamine in a 5% CO2 environment. Approximately 80% confluent cultures are harvested by brief trypsinization (0.0625% trypsin-0.25 mM EDTA in Cell Dissociation Medium) (Life Technologies) and resuspended at a final concentration of 8×10^7 cells per ml in magnesium and calcium free phosphate buffered saline for implantation. Cell viability is determined by Trypan blue exclusion and found to be >95%.

On the day of implantation, animals are anesthetized with either isoflurane or Ketaset and Rompun and the abdomen is prepared for sterile surgery. A small abdominal incision is made and the ascending colon identified. The gut is then placed on strips of sterile gauze before injection. Two million viable tumor cells in 0.025 ml PBS are injected under the serosa into the muscularis/submuscularis by means of a sterile tuberculin syringe and a 27 gauge needle. Cells are injected so as to visibly infiltrate between the submucosal and subserosal tissues. The serosal surface at the injection site is dabbed gently with 70% isopropyl alcohol pads to kill tumor cells that may have escaped. The organs are replace *in situ*. The abdominal wall is closed with continuous nylon sutures. The outer skin is then closed using wound clips which are removed seven days post implantation.

To ensure that cell implantation is properly performed, after 7 days, several control animals are euthanized by cerebral dislocation , the abdominal organs and thorax examined for the presence of macroscopic "primary" colonic tumors and metastases. Pilot studies demonstrated that at this time intracolonic tumors of approximately 5 to 7 mm^3 are present without peritoneal spread of tumor after injection of C6 cells. One day following implantation of cells, animals are treated once daily intraperitoneally with either MCTA at 20 mg/kg/day in VPD:D5W or vehicle alone in a 0.1 ml bolus. The health of the animals is monitored daily and if signs of severe discomfort or pain is observed or the animal is deemed to be moribund, animals are sacrificed humanely. Dosing of the animals continued until all surviving animals in the experiment are deemed moribund. When possible, the local tumor growth on the colon is measured and the major organs such as lung, heart, spleen, liver and kidney are resected from moribund animals and submitted for histopathological analysis.

B. MCTA against FGF/FGFR

The examples below are non-limiting and are merely representative of various aspects and features of the present invention. They demonstrate that the compositions herein described inhibit the activity of FGFR in cells and can be used to reduce tumor growth and inhibit metastasis.

4. Inhibition of FGFR-Stimulated DNA Synthesis

The following example illustrates the ability of MCTA to inhibit ligand-stimulated DNA synthesis in cells, specifically when stimulated with FGF or PDGF but not when stimulated with EGF. DNA synthesis is required for many of the activities of FGFR including, but not limited to, cell proliferation. Uridine is added to remove the effect of a metabolite of MCTA. (See Greene, et al., Biochem. Pharmacol. 50(6):861, 1995; Nair, et al., Immunology Letters 47:171, 1995)

Materials And Methods

(1) EGF: mouse EGF, 201; Toyobo,Co., Ltd. Japan, PDGF, Boehringer Mannheim, Germany, FGF, Gibco, USA

(2) BrdU Labeling Reagent: 10 mM, in PBS (pH7.4),Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(3) FixDenat: fixation solution (ready to use), Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(4) Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(5) TMB Substrate Solution: tetramethylbenzidine (TMB), ready to use, Cat. No. 1 647 229, Boehringer Mannheim, Germany.

(6) PBS Washing Solution : 1X phosphate buffered saline, pH 7.4

(7) Albumin, Bovine (BSA): fraction V powder; A-8551, Sigma Chemical Co., USA.

(8) NIH3T3 clone C7 (3T3/EGFRc7)(Honegger et al., Cell 51:199-209, 1987)) engineered to over-express human EGF receptor. These cells natively express FGFR and PDGFR.

Protocol

(1) 3T3/EGFRc7 cells are seeded at 8000 cells/well in DMEM, 10% CS, 2mM Gln in a 96 well plate. Cells are incubated overnight at 37 °C in 5% CO_2.

(2) After 24 hours, the cells are washed with PBS, and then serum starved in serum free medium (0% CS DMEM with 0.1% BSA) for 24 hours.

(3) On day 3, ligand (2nM EGF, 3.8 nM PDGF or 1.5 nM bFGF) prepared in DMEM with 0.1% BSA and 30 µM (final concentration) uridine) and test compound was added to the cells simultaneously. The negative control wells received serum free DMEM with 0.1% BSA only; the positive control cells received ligand (EGF, PDGF or FGF) but no test compound. Test compound was prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.

(4) After 20 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) was added and the cells are incubated with BrdU (final concentration=10 µM) for 1.5 hours.

(5) After incubation with labeling reagent, the medium was removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution was added (50 µl/well) and the plates incubated at room temperature for 45 minutes on a plate shaker.

(6) The FixDenat solution was thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate

was incubated for 30 minutes at room temperature on a plate shaker.

(7) The blocking solution was removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) was added (100 µl/well) and the plate was incubated for 90 minutes at room temperature on a plate shaker.

(8) The antibody conjugate was thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate dried by inverting and tapping on a paper towel.

(9) TMB substrate solution was added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development was sufficient for photometric detection.

(10) The absorbance of the samples was measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

Results

The results shown in Table 4 below demonstrate that MCTA inhibits the specific biological response of cells to FGF while having no effect on the response of the cells to EGF. MCTA also inhibits the specific biological responses to PDGF. The results are shown as IC50s, the concentration at which 50% of the maximal BrdU incorporation response is inhibited.

Table 4

	FGF-induced DNA synthesis (IC50 uM)	**PDGF-induced DNA synthesis (IC50 µM)**	**EGF-induced DNA synthesis (IC50 uM)**
Cell Line/ Ligand	**EGFRC7/FGF**	**EGFRC7/PDGF**	**EGFRC7/EGF**
MCTA	80	45	>100

5. Inhibition of Tumor Growth *in vivo*

The following example demonstrates the ability of MCTA to inhibit the in vivo growth of tumors characterized by inappropriate FGFR activity while having no inhibitory effect on a tumor that does not express FGFR. The FGFR expressing cells are from a prostate cancer (PC3, Nakamoto, et al., Cancer Res. 52:571, 1992). The non-FGFR-expressing cell line was obtained from an epidermoid carcinoma (A431, ATTC CRL 1555, Takahashi, et al., supra).

Materials And Methods

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They received sterile rodent chow and water ad libitum.

Cell lines are grown in appropriate medium (A431 - DMEM with 10% FBS and 2 mM GLN; PC3 - HAM'S F12 with 7% FBS and 2 mM GLN). All cell culture media, glutamine, and fetal bovine serum is purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group). Tumor growth was measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students' t-test. MCTA in 50 - 100 µL excipient (DMSO, or VPD:D5W) was delivered by IP injection at different concentrations.

Results

The results of the subcutaneous xenograft experiments, shown in Table 5 below, demonstrate that administration of MCTA significantly inhibited the growth of tumors associated with FGFR activity with no significant effect on a non-FGFR expressing tumor demonstrating MCTA's effectiveness as a treatment for cancers characterized by inappropriate FGFR activity.

Table 5

Cell line	Dose	% Inhibition	P value
PC-3	20mg/kg/day	71%	$\leq$0.01
A431	14mg/kg/day	40%	not sig.

6. Inhibition of Tumor Growth and Metastasis *in vivo*

The following example illustrates the ability of MCTA to inhibit growth and metastasis of a tumor cell line expressing FGFR (C6 cells).

Materials And Methods

Ten to 12 week old athymic Balb/c nu/nu mice are obtained from Simonsen Laboratory (Gilroy, CA) and maintained in a pathogen-free environment throughout the experiments.

C6 cells (ATCC CCL 107) are grown and maintained in F-10 medium (Life Technologies, Inc. Grand Island, NY) supplemented with 10% fetal bovine serum, 2mM glutamine in a 5% CO2 environment. Approximately 80% confluent cultures are harvested by brief trypsinization (0.0625% trypsin-0.25 mM EDTA in Cell Dissociation Medium) (Life Technologies) and resuspended at a final concentration of 8×10^7 cells per ml in magnesium and calcium free phosphate buffered saline for

implantation. Cell viability was determined by Trypan blue exclusion and found to be >95%.

On the day of implantation, animals are anesthetized with either isoflurane or Ketaset and Rompun and the abdomen was prepared for sterile surgery. A small abdominal incision was made and the ascending colon identified. The gut was then placed on strips of sterile gauze before injection. Two million viable tumor cells in 0.025 ml PBS is injected under the serosa into the muscularis/submuscularis by means of a sterile tuberculin syringe and a 27 gauge needle. Cells are injected so as to visibly infiltrate between the submucosal and subserosal tissues. The serosal surface at the injection site was dabbed gently with 70% isopropyl alcohol pads to kill tumor cells that may have escaped. The organs are replace *in situ*. The abdominal wall was closed with continuous nylon sutures. The outer skin was then closed using wound clips which are removed seven days post implantation.

To ensure that cell implantation was properly performed, after 7 days, several control animals are euthanized by cerebral dislocation , the abdominal organs and thorax examined for the presence of macroscopic "primary" colonic tumors and metastases. Pilot studies demonstrated that at this time intracolonic tumors of approximately 5 to 7 mm^3 are present without peritoneal spread of tumor after injection of C6 cells.

One day following implantation of cells, animals are treated once daily intraperitoneally with either MCTA at 20 mg/kg/day in VPD:D5W or vehicle alone in a 0.1 ml bolus. The health of the animals is monitored daily and if signs of severe discomfort or pain is observed or the animal is deemed to be moribund, animals are sacrificed humanely. Dosing of the animals continued until all surviving animals in the experiment are deemed moribund. When possible, the local tumor growth on the colon is measured and the major organs such as lung, heart, spleen, liver and kidney are resected from moribund animals and submitted for histopathological analysis.

Results

In this model, vehicle treated animals are found to be all moribund by day 16 - day 18 with a 50% survival rate at approximately day 12 post implantation. Gross observation of moribund animals in this group showed severe metastasis in the liver, occasional tumor growth in the spleen, occasional carcinomatosis (metastasis in the peritoneal cavity) and the presence of a richly vascularized local tumor.

In two separate experiments, it was found that daily treatment with MCTA at 20 mg/kg/day extended the survival of the animals by >200% with a 50% survival rate at day 30 - day 33 post-implantation. Necropsy of a selected moribund animal showed several nodules of metastasis in the liver and an apparently pale while local tumor, suggesting that treatment with MCTA had an antiangiogenic effect. In comparison to the vehicle treated animals, an >87% inhibition of local tumor growth was observed in animals treated with MCTA. Histopathology results confirmed the presence of limited liver metastasis with no apparent metastasis to the brain.

C. MCTA/BCNU

Materials: BCNU is commercially available. One source is Bristol Laboratories Oncology Products, Princeton, NJ, under the trade name BiCNU®.

A synthetic procedure amenable to the preparation of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is described in US Patent No. 4,087,535 which is incorporated by reference as if fully set forth herein.

Procedure: SF763T cells, derived from human glioblastoma cells, are used in all of the examples shown below. $1X10^7$ cells per mouse are implanted in 100 µl PBS subcutaneously into the hindflanks of female BALC/c nu/nu mice. Tumors are allowed to establish before dosing was initiated.

N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide was administered IP in VDP:D5W (VDP: 3% benzyl

alcohol (w/v), 8% Tween 80 (v/v), 65% PEG 300 (w/v), QS to 100% with 100% ethanol; D5W: 5% dextrose (w/v) in water) at 2.5, 5.0, 10 and 20 mg/kg from day 8 to day 29 post implantation. BCNU was administered IV in 2% ethanol/saline at 8, 12, 18 and 27 mg/kg on days 8, 12 and 16 post implantation. Combinations of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide at 2.5, 5.0 and 10 mg/kg and BCNU at 8, 12 and18 mg/kg were also administered with the same regimens.

Tumor weight is determined twice per week until day 43 post implantation. In general the size of the tumor is determined by measuring two of its dimensions, length and width, by deducing its depth using procedures well known to those skilled in the art, and calculating its weight based on standard assumptions regarding the density of such tumors.

Example 7

Example 1, the results of which can be seen in graphical form as the CONTROL in each of Figures 1-6 shows tumor growth in untreated mice. All subsequent examples are compared to this control for determination of whether statistically significant inhibition is being shown by either N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide or BCNU alone or by various combinations of these two drugs.

Example 8

This example, shown graphically in Figure 1, shows the effect of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone at 20 mg/kg dose. Analysis of the raw data obtained in this example (not shown) demonstrated that while N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is being administered, statistically significant inhibition of tumor growth is observed. When treatment with N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is stopped, tumor growth resumed. This demonstrates that N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is in

fact cytostatic rather than cytotoxic; i.e., N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide temporarily stops cell activity but does not kill the cell.

Example 9

This example, the results of which are also shown graphically in Figure 1, shows the results of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone at 10 mg/kg. Again, statistical analysis of the raw data, not shown, revealed that the percentage inhibition is less than with 20 mg/kg but inhibition continued throughout the time period during which N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is being actively administered and then, when treatment with N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide ceased, tumor growth resumed.

Example 10

This example, the results of which are likewise shown in Fig. 1 shows the effect of 5 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide. Again, the percentage inhibition is less but, when the raw data is statistically analyzed using the Student's T Test, still statistically significant.

Example 11

At 2.5 mg/kg, N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide, the raw data did not show statistically significant inhibition of tumor growth. This is graphically depicted in Fig.1 where it can be seen that the curve for 2.5 mg/kg essentially tracks that of the control.

Example 12

Here, the effect of 27 mg/kg of BCNU alone is shown. One dose at this rate is effective to maintain a relatively high

percentage inhibition of tumor growth for the duration of this test. This is graphically depicted in Fig. 2.

Example 14

At 18 mg/kg, BCNU is already losing some of its potency. Analysis of the raw data (not shown) revealed statistically significant percentages inhibition only up to day 22 at which time tumor growth began to catch up with the control. Again, this is shown graphically in Fig.2.

Example 14

At 12 mg/kg BCNU is found to be ineffective; i.e., no statistically significant percentage inhibition is observed from the raw data. Graphically, it can be seen in Fig. 2 that the 12 mg/kg curve shows some inhibition in the days immediately post treatment but as the days post-treatment increassed, the curve approached that of the control.

Example 15

At 8 mg/kg, BCNU had no therapeutic effect; i.e. in Fig. 2 the curve for 8 mg/kg essentially tracks that of the control.

Example 16

When N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is administered at 10 mg/kg and BCNU at 18 mg/kg, the result is very positive. Statistical analysis of the raw data (not shown) showed that the overall percentage inhibition is substantially higher than treatment with BCNU alone at 18 mg/kg or N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide alone at 10 mg/kg. Furthermore, the time period over which statistically significant inhibition occurred is much longer than that observed with either drug alone. Fig. 3 graphically displays this result.

Example 17

In this example the combination of 10 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide and 12 mg/kg BCNU is quite effective; the raw data showed statistically significant inhibition up to day 25 of the trial. To the contrary, 12 mg/kg BCNU alone showed no statistically significant effect. This is graphically depicted in Fig. 4. This ability to achieve inhibition as lower doses of BCNU should be of great benefit to patients.

Example 18

When 18 mg/kg BCNU is augmented with only 5 mg/kg N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide, not only is a higher percentage inhibition realized over 18 mg/kg of BCNU alone, the duration of statistically significant inhibition is longer, up to day 29 as opposed to only day 22 with BCNU alone indicating that even at very low doses the N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is having a marked positive effect on the efficacy of BCNU. This is shown graphically in Fig. 5.

Example 19

The graphical representation of the combination of 5 mg/kg of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide with 12 mg/kg BCNU showed some marginal effectiveness but statistical analysis of the raw data did not attach any statistical significance to the result.

Conclusion

Thus, it will be appreciated that the compounds, methods and pharmacological compositions of the present invention modulate PTK activity and therefore are expected to be effective as therapeutic agents against PTK-related disorders.

It will also be appreciated that the heteroaryl-carboxamide, N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-

carboxamide ("MCTA"), is particularly useful for the treatment of disorders characterized by inappropriate FGFR activity and is expected to be especially useful in the treatment of invasive cancers noted for their aggressiveness and tendency to metastatize.

Finally, it will be appreciated N-(4-trifluoromethyl-phenyl)-5-methylisoxazole-4-carboxamide together with a chemotherapeutic nitrosourea, in particular BCNU, is an effective tumor suppressant for human glioblastoma tumor cells at dosages substantially lower than that necessary when either of the compounds is used alone and that it is therefore expected that this combination will have similarly positive results in human subjects.

Although certain embodiments and examples have been used to describe the present invention, it will be apparent to those skilled in the art that changes to the embodiments and examples shown may be made without departing from the scope and spirit of the invention.

Other embodiments are within the following claims.

Claims

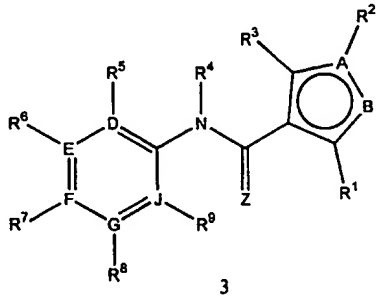
1. A heteroarylcarboxamide compound, or physiologically acceptable salt thereof, which modulates the activity of a protein tyrosine kinase.

2. The compound of claim 1 wherein said protein tyrosine kinase comprises a receptor tyrosine kinase.

3. The compound of claim 2 wherein said receptor tyrosine kinase comprises a fibroblast growth factor receptor (FGFR).

4. The compound of claim 2 wherein said receptor tyrosine kinase comprises a platelet derived growth factor receptor (PDGFR).

5. The compound of claim 1 having the following chemical structure:



3

wherein:

A is selected from the group consisting of oxygen, nitrogen and sulfur;

B is selected from the group consisting of nitrogen and sulfur and it is understood that when B is sulfur and A is nitrogen, said nitrogen is participating in both a single bond and a double bond within the ring so that it cannot be bonded to any atom outside the ring; that is, when B is sulfur, R^2 does not exist;

D, E, F, G, and J are independently selected from the group consisting of carbon and nitrogen and, furthermore, when any of D, E, F, G and J is nitrogen, R^5, R^6, R^7, R^8 and R^9, respectively, does not exist;

R^1 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl and heteroalicyclic;

R^2 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl, heteroalicyclic, carbonyl, C-carboxy, S-sulfonamido, sulfonyl, hydroxy, alkoxy, trihalomethanesulfonyl, halo, guanyl, C-amido and C-thioamido;

R^3 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl, heteroaryl and heteroalicyclic;

Z is selected from the group consisting of oxygen and sulfur;

R^4 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, alkenyl, alkynyl, aryl heteroaryl, heteroalicyclic, sulfonyl, trihalomethanesulfonyl, hydroxy, alkoxy and C-carboxy;

R^5, R^6, R^7, R^8 and R^9 are independently selected from the group consisting of hydrogen, alkyl, trihaloalkyl, alkenyl, alkynyl, cycycloalkyl, aryl, heteroaryl, heteroalicyclic , hydroxy, alkoxy, cycloalkoxy, aryloxy, heteroaryloxy, heteroalicycloxy, thiohydroxy, thioalkyoxy, thiocycloalkoxy, thioheteraryloxy, thioheteralicycloxy, halo, nitro, cyano, O-carbamyl, N-carbamyl, O-thiocarbamyl, N-thiocarbamyl, silyl, phosphonyl, C-carboxy, O-carboxy, N-amido, C-amido, sulfinyl, sulfonyl, S-sulfonamido, N-sulfonamido, trihalomethanesulfonyl, guanyl, guanidino, trihalomethanesulfonamido, amino and $-NR^{14}R^{14}$, wherein,

R^{14} and R^{14} are independently selected from the group consisting of hydrogen, alkyl, cycloalkyl, aryl, carbonyl, C-carboxy, sulfonyl, trihalomethanesulfonyl and, combined, a

five- or six-member heteroalicyclic ring containing at least one nitrogen;

and physiologically acceptable salts thereof.

6. The compound or salt of claim 5 wherein A is oxygen and B is nitrogen.

7. The compound or salt of claim 6 wherein R^1 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, aklenyl and alkynyl.

8. The compound or salt of claim 7 wherein R^3 is selected from the group consisting of hydrogen, alkyl, cycloalkyl and aryl.

9. The compound or salt of claim 8 wherein R^4 is hydrogen.

10. The compound or salt of claim 9 wherein R^2 is selected from the group consisting of hydrogen, alkyl an cycloalkyl.

11. The compound or salt of claim 10 wherein Z is oxygen.

12. The compound or salt of claim 11 wherein R^5, R^6, R^8 and R^9 are hydrogen.

14. The compound or salt of claim 12 wherein R^7 is selected from the group consisting of trihalomethyl and trihalomethanesulfonyl.

14. The compound or salt of claim 14 wherein R^5, R^8 and R^9 are hydrogen.

15. The compound or salt of claim 14 wherein R^6 and R^7 combine to form a methylenedioxy or a 1,3-dioxano group.

16. The compound or salt of claim 5 wherein A and B are nitrogen.

17. The compound or salt of claim 16 wherein R^1 is selected from the group consisting of hydrogen, alkyl, cycloalkyl, aklenyl and alkynyl.

18. The compound or salt of claim 17 wherein R^3 is selected from the group consisting of hydrogen, alkyl, cycloalkyl and aryl.

19. The compound or salt of claim 18 wherein R^4 is hydrogen.

20. The compound or salt of claim 19 wherein R^2 is selected from the group consisting of hydrogen, alkyl an cycloalkyl.

21. The compound or salt of claim 20 wherein Z is oxygen.

22. The compound or salt of claim 21 wherein R^5, R^6, R^8 and R^9 are hydrogen.

23. The compound or salt of claim 22 wherein R^7 is selected from the group consisting of trihalomethyl and trihalomethanesulfonyl.

24. The compound or salt of claim 21 wherein R^5, R^8 and R^9 are hydrogen.

25. The compound or salt of claim 24 wherein R^6 and R^7 combine to form a methylenedioxy or a 1,3-dioxano group.

26. The compound or salt of claim 5 wherein J is nitrogen.

27. The compound or salt of claim 26 wherein:

R^7 is selected from the group consisting of trihalomethyl and trihalomethanesulfonyl; and,

R^5, R^6, R^8, and R^9 are hydrogen.

28. The compound or salt of claim 27 wherein R^1 is selected from the group consisting of hydrogen, alkyl and cycloalkyl.

29. The compound or salt of claim 28 wherein R^3 is selected from the group consisting of hydrogen, alkyl and aryl.

30. A method for the treatment or prevention of a disorder characterized by inappropriate protein tyrosine kinase activity comprising administering to an organism afflicted with such a disorder a therapeutically effective amount of one of more of said compounds of claim 1 or a physiologically acceptable salt thereof.

31. A pharmacological composition of said compound of claim 1.

32. The method of claim 30 wherein said therapeutically effective amount of said compound of claim 1 is administered in said pharmacological composition.

33. The method of claim 30 wherein said organism comprises a mammal.

34. The method of claim 33 wherein said mammal is a human.

35. The method of claim 30 wherein said disorder comprises cancer.

36. The method of claim 35 wherein said cancer comprises brain cancer, colon cancer, prostate cancer, kidney cancer, breast cancer, lung cancer, salivary gland cancer, oral cancer, pancreatic cancer, bladder cancer, Kaposi's sarcoma, melanoma and ovarian cancer.

37. The method of claim 30 wherein said disorder comprises a skeletal disorder.

38. The method of claim 30 wherein said disorder comprises a fibrotic disorder.

39. The method of claim 30 wherein said disorder comprises a blood vessel proliferative disorder.

40. The method of claim 38 wherein said fibrotic disorder comprises arterial restenosis, liver cirrhosis, kidney sclerosis and wound scarring.

41. A method of inhibiting the metastasis of a cancer comprising administering to said organism said pharmacological composition of said compound of claim 1.

42. The method of claim 41 wherein said cancer comprises colon cancer, prostate cancer, pancreatic cancer, Kaposi's sarcoma, ovarian cancer, breast cancer and gliomas.

43. A method of treating a disorder characterized by inappropriate FGFR activity in a patient comprising the step

of administering to said patient a therapeutically effective amount of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide in a pharmaceutically acceptable carrier.

44. The method of claim 43 wherein the disorder is cancer.

45. The method of claim 44 wherein the cancer is selected from the group consisting of brain cancer, colon cancer, prostate cancer, kidney cancer, breast cancer, salivary gland cancer, oral cancer, pancreatic cancer, bladder cancer, Kaposi's sarcoma and ovarian cancer.

46. The method of claim 45 wherein the cancer is selected from the group consisting of prostate cancer, pancreatic cancer, kidney cancer and bladder cancer.

47. The method of claim 43 wherein the pharmaceutically acceptable carrier is VPD:D5W.

48. The method of claim 43 wherein the disorder is benign prostatic hypertrophy.

49. The method of claim 43 wherein the disorder is a skeletal disorder.

50. The method of claim 43 wherein the disorder is a fibrotic disorder.

51. The method of claim 50 wherein the fibrotic disorder is selected from the group consisting of hepatic cirrhosis, glomular sclerosis, interstitial nephritis, interstitial pulmonary fibrosis, restinosis, atherosclerosis, wound scarring and scleroderma.

52. A method of inhibiting the metastasis of a cancer characterized by inappropriate FGFR activity comprising administering to a patient N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide in a pharmaceutically acceptable carrier.

53. The method of claim 52 wherein the cancer is selected from the group consisting of colon cancer, prostate cancer, pancreatic cancer and Kaposi's sarcoma.

54. A method for inhibiting tumorigenic activity in a cell by administering to said cell a chemotherapeutic nitrosourea and N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide.

55. The method of claim 54 wherein said chemotherapeutic nitrosourea comprises 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU).

56. The method of claim 55 wherein said tumorigenic activity comprises a malignant neoplasm.

57. The method of claim 56 wherein said malignant neoplasm comprises a brain tumor.

58. The method of claim 57 wherein said brain tumor comprises a primary intra-axial brain tumor.

59. The method of claim 58 wherein said intra-axial brain tumor comprises a glioblastoma.

60. The method of claim 58 wherein said primariy intra-axial brain tumor comprises an astrocytoma.

61. A method for the treatment of a solid tumor in a patient by administering to said patient in need of such treatment a therapeutically effective amount of a chemotherapeutic nitrosourea and a therapeutically effective amount of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide.

62. The method of claim 61 wherein said chemotherapeutic nitrosourea comprises BCNU.

63. The method of claim 62 wherein said therapeutically effective amount of said BCNU comprises a first pharmacological composition.

64. The method of claim 61 wherein said therapeutically effective amount of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide comprises a second pharmacological composition.

65. The method of claim 64 wherein said therapeutically effective amount of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide comprises an initial loading dose.

66. The method of claim 65 wherein said initial loading dose comprises administration of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide to said patient over an interval of about 3 to about 6 days.

67. The method of claim 66 wherein said initial loading dose comprises administration of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide in equal amounts on each of said days of said interval.

68. The method of claim 66 wherein said initial loading dose comprises administration of said N-(4-trifluoromethyl-

phenyl)-5-methylisoxazole-4-carboxamide as a continuous infusion over said 3 - 6 day interval.

69. The method of claim 67 or 68 wherein said initial loading dose comprises from about 350 mg/m^2/day to about 450 mg/m^2/day.

70. The method of claim 69 wherein on the day immediately following the last day of said administration of said initial loading dose of N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide to said patient, said therapeutically effective dose of said BCNU is administered to said patient.

71. The method of claim 70 wherein said therapeutically effective dose of BCNU comprises from about 50 mg/m^2 to about 200 mg/m^2.

72. The method of claim 71 wherein subsequent to said loading dose, a maintenance dose of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide is administered to said patient.

73. The method of claim 72 wherein said maintenance dose of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide comprises from about 350 mg/m^2 to about 450 mg/m^2.

74. The method of claim 73 wherein from about 10 to about 15 additional of said maintenance dose is administered to said patient.

75. The method of claim 74 wherein each said additional maintenance dose is administered to said patient at intervals of from 5 to about 10 days after each previous said maintenance dose.

76. The method of claim 75 wherein each said additional maintenance dose comprises from about 350 mg/m^2/week to about 450 mg/m^2/week of said N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide.

77. The method of claim 61 wherein said solid tumor comprises a malignant neoplasm.

78. The method of claim 77 wherein said malignant neoplasm comprises a brain tumor.

79. The method of claim 78 wherein said brain tumor comprises a primary intra-axial brain tumor.

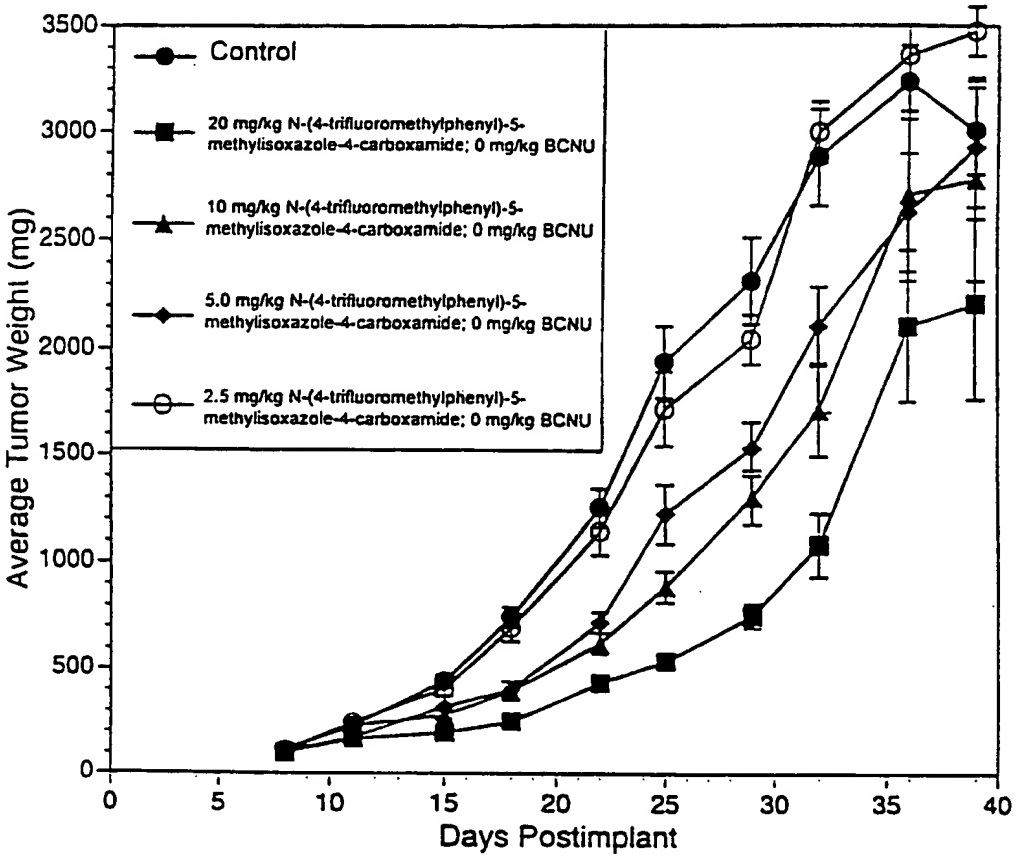
80. The method of claim 79 wherein said primary intra-axial brain tumor comprises a glioblastoma.

81. The method of claim 79 wherein said primary intra-axial brain tumor comprises an astrocytoma.

82. The method of claim 61 wherein said patient is a mammal.

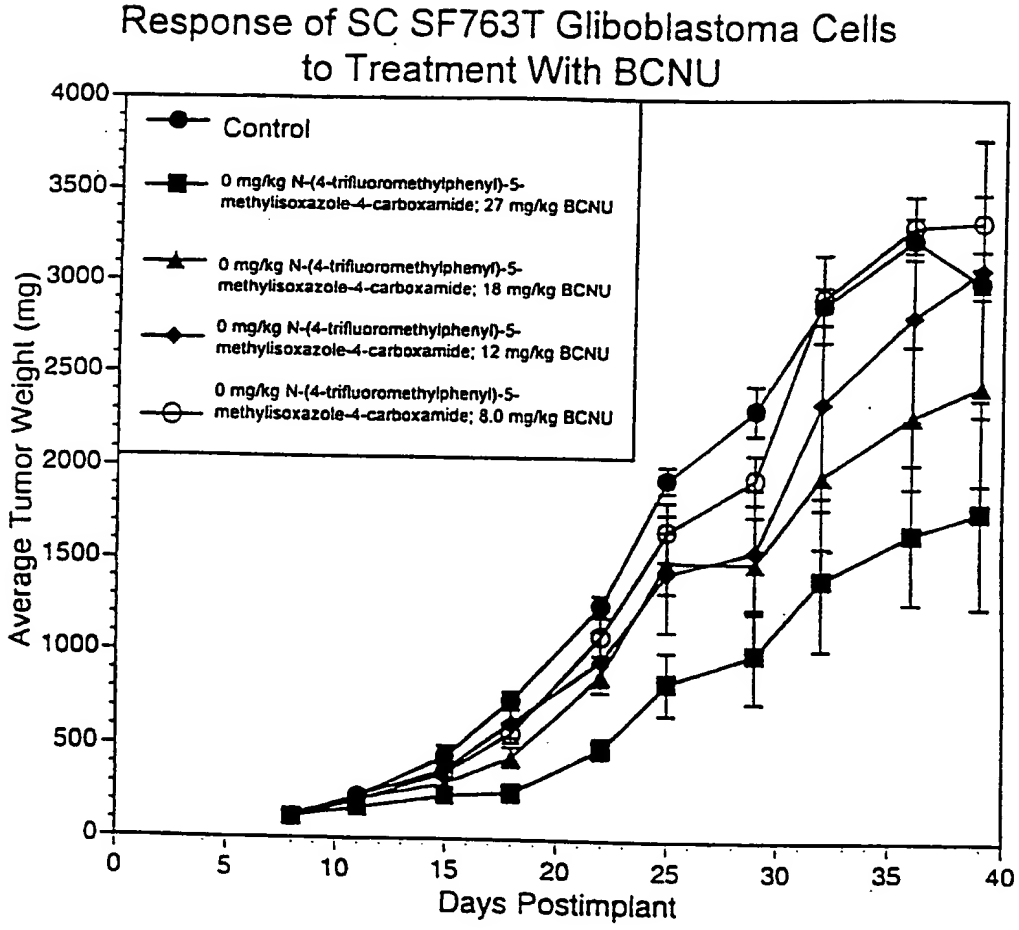
83. The method of claim 82 wherein said mammal is a human being.

Response of SC SF763T Gliboblastoma Cells to Treatment With N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide



N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide dose response
Dosing on days 8, 12 and 16

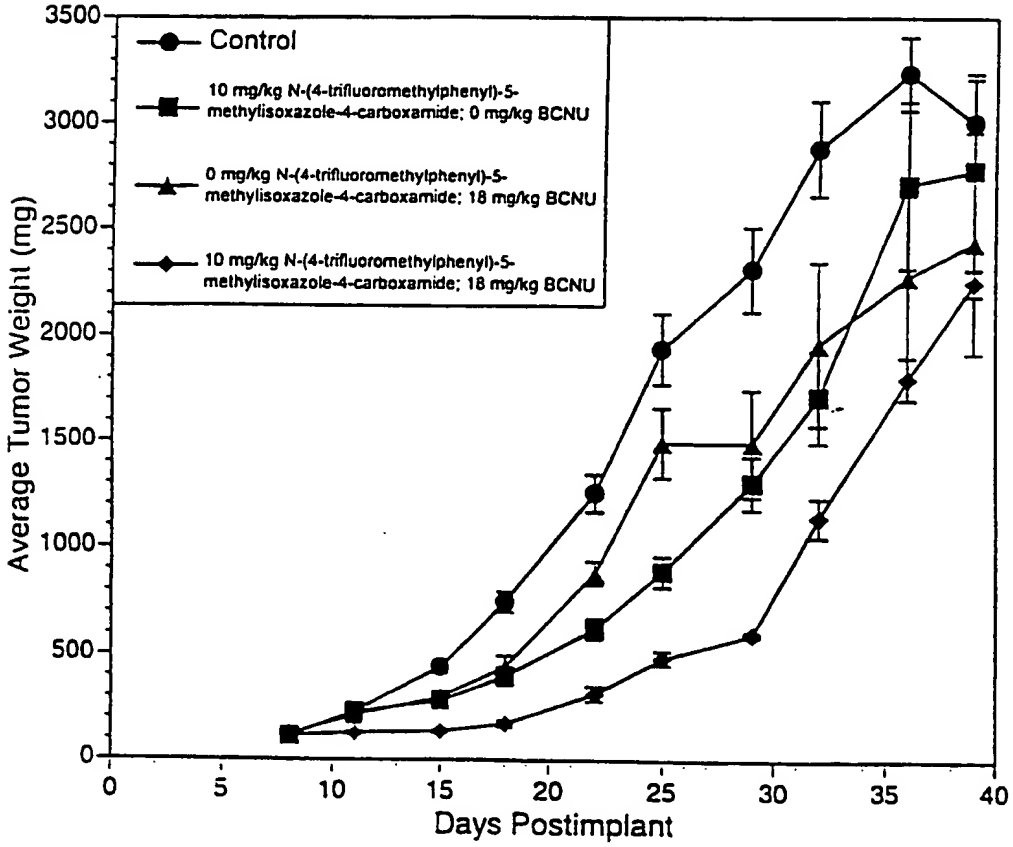
Figure 1



BCNU dose response
Dosing on days 8, 12 and 16

Figure 2

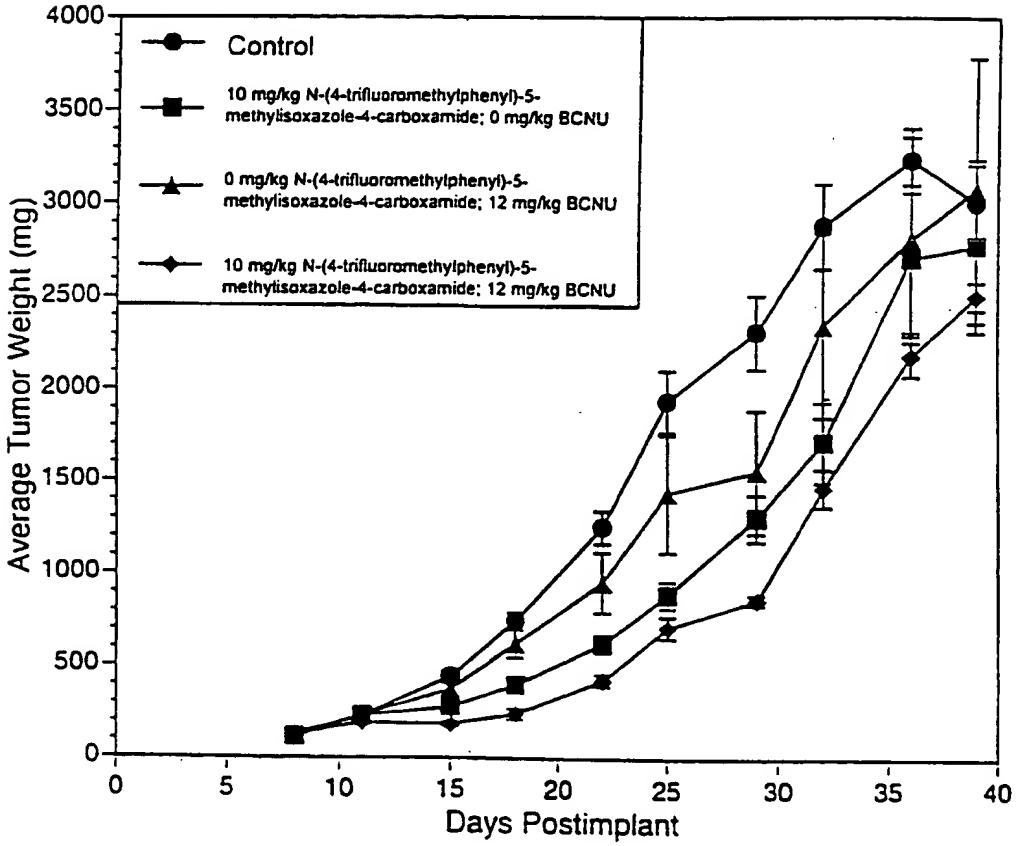
Response of SC SF763T Gliboblastoma Cells to Treatment With N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide and BCNU



N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide at 10 mg/kg/day days 8 to 29
BCNU at 18 mg/kg on days 8, 12 and 16

Figure 3

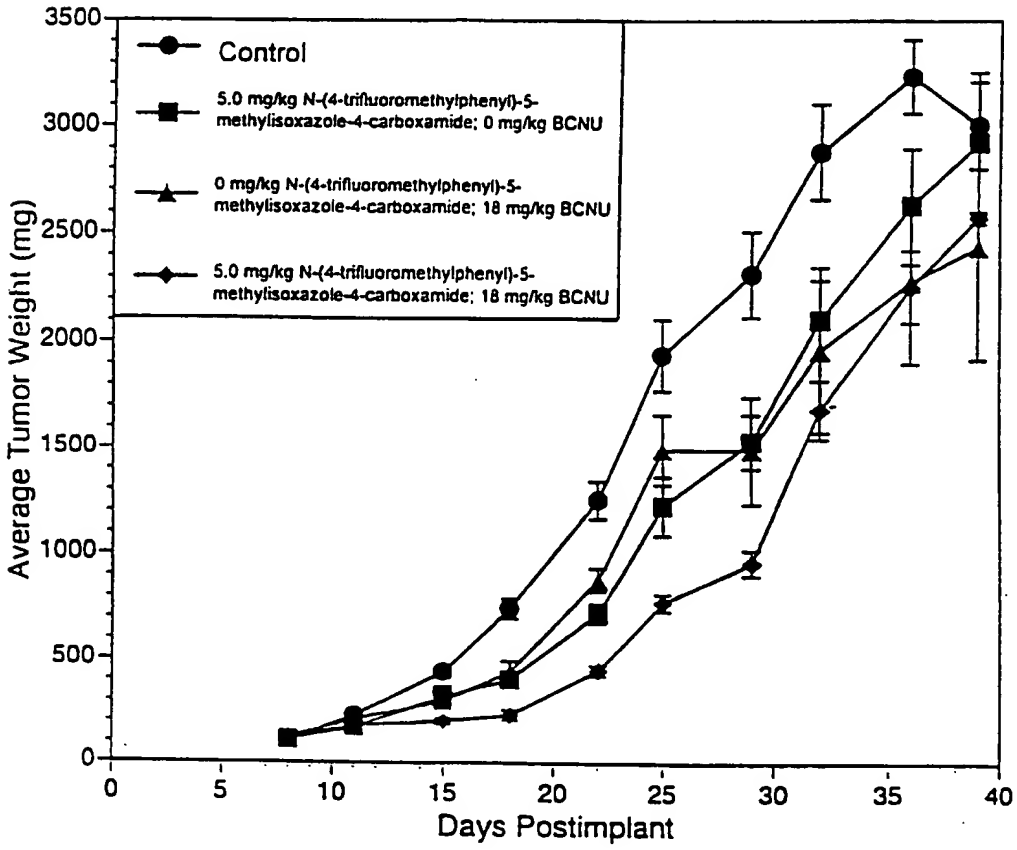
Response of SC SF763T Gliboblastoma Cells to Treatment With N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide and BCNU



N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide at 10 mg/kg/day days 8 to 29
BCNU at 12 mg/kg on days 8, 12 and 16

Figure 4

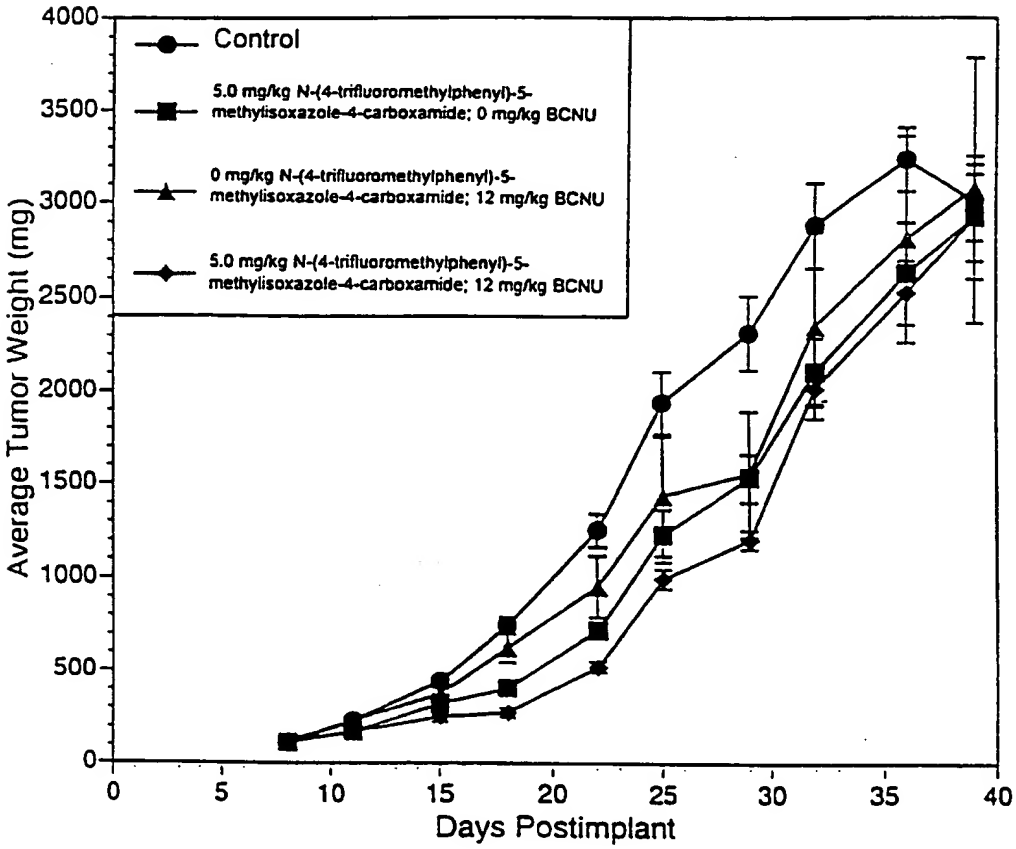
Response of SC SF763T Gliboblastoma Cells to Treatment With N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide and BCNU



N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide at 5.0 mg/kg/day days 8 to 20
BCNU at 18 mg/kg on days 8, 12 and 16

Figure 5

Response of SC SF763T Gliboblastoma Cells to Treatment With N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide and BCNU



N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide at 5.0 mg/kg/day days 8 to 29
BCNU at 12 mg/kg on days 8, 12 and 16

Figure 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10174

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07D 413/02, 231/12, 261/18, 261/08
US CL : 546/272.1, 275.4; 548/374.1, 375.1, 376.1, 248

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 546/272.1, 275.4; 548/374.1, 375.1, 376.1, 248

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,700,823 A (HIRTH et al.) 12 December 1997, abstract, fig. 1A.	1-5, 31-84
X, Y, E	US 5,789,427 A (CHEN et al.) 04 August 1998, abstract, fig. 4D, fig. 4A.	1-5, 31-84

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 AUGUST 1998	Date of mailing of the international search report 03 NOV 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DOMINIC KEATING Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10174

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 6-30
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 6 is missing. Claims 7-30 depend from claim 6.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-5 and 31-84 to the extent that they read on Groups I, II, IV and V

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10174

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, the invention of claim 1, wherein the six membered ring contains phenyl, and the five membered ring contains isoxazole.

Group II, the invention of claim 1, wherein the six membered ring contains phenyl, and the five membered ring contains pyrazole.

Group III, the invention of claim 1, wherein the six membered ring contains phenyl, and the five membered ring contains thiazole.

Group IV, the invention of claim 1, wherein the six membered ring contains pyridyl and the five membered ring contains isoxazole.

Group V, the invention of claim 1, wherein the six membered ring contains pyridyl and the five membered ring contains pyrazole.

Group VI, the invention of claim 1, wherein the six membered ring contains pyridyl and the five membered ring contains thiazole.

Group VII, the invention of claim 1, wherein the six membered ring contains benzodioxyl and the five membered ring contains isoxazole.

Group VIII, the invention of claim 1, wherein the six membered ring contains benzodioxyl and the five membered ring contains pyrazole.

Group IX, the invention of claim 1, wherein the six membered ring contains benzodioxyl and the five membered ring contains thiazole.

Claims 2-5 and 31-84 will be examined as commensurate in scope with the group elected.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: they do not share the same essential structural element(s) that define the "special technical feature" necessary to specify a contribution over the prior art. The structural moiety common to all the groups is a carboxamide which is known in the art and, therefore, cannot be said to be the special technical feature which makes a contribution over the prior art. All other substituents differ structurally from each other, e.g., the phenyl and the pryidyl substitutents, each of which are known in the prior art. Thus, these claims lack the corresponding special technical feature(s) necessary to link them together to fulfill the Unity of Invention requirement.